

60 REC OF PCT/PTO 17 SEP 2001

FORM PTO-1390 (Modified)
(REV 11-2000)

U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

ATTORNEY'S DOCKET NUMBER

TRANSMITTAL LETTER TO THE UNITED STATES

213930US0PCT

DESIGNATED/ELECTED OFFICE (DO/EO/US)

U.S. APPLICATION NO. (IF KNOWN, SEE 37 CFR

CONCERNING A FILING UNDER 35 U.S.C. 371

09/926163

INTERNATIONAL APPLICATION NO

INTERNATIONAL FILING DATE

PRIORITY DATE CLAIMED

PCT/JP00/01608

16 March 2000

17 March 1999 (earliest)

TITLE OF INVENTION

SORBITOL DEHYDROGENASE, GENE ENCODING THE SAME AND USE THEREOF

APPLICANT(S) FOR DO/EO/US

Takashi SHIBATA, et al

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☒ This is an express request to begin national examination procedures (35 U.S.C. 371(f)). The submission must include items (5), (6), (9) and (24) indicated below.
4. ☒ The US has been elected by the expiration of 19 months from the priority date (Article 31).
5. ☒ A copy of the International Application as filed (35 U.S.C. 371 (c) (2))
 - a. ☐ is attached hereto (required only if not communicated by the International Bureau).
 - b. ☒ has been communicated by the International Bureau.
 - c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☐ An English language translation of the International Application as filed (35 U.S.C. 371(c)(2)).
 - a. ☐ is attached hereto.
 - b. ☐ has been previously submitted under 35 U.S.C. 154(d)(4).
7. ☒ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371 (c)(3))
 - a. ☐ are attached hereto (required only if not communicated by the International Bureau).
 - b. ☐ have been communicated by the International Bureau.
 - c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
 - d. ☒ have not been made and will not be made.
8. ☐ An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. ☐ An oath or declaration of the inventor(s) (35 U.S.C. 371 (c)(4)).
10. ☐ An English language translation of the annexes of the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371 (c)(5)).
11. ☒ A copy of the International Preliminary Examination Report (PCT/IPEA/409).
12. ☒ A copy of the International Search Report (PCT/ISA/210).

Items 13 to 20 below concern document(s) or information included:

13. ☐ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
14. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
15. ☐ A **FIRST** preliminary amendment.
16. ☐ A **SECOND** or **SUBSEQUENT** preliminary amendment.
17. ☐ A substitute specification.
18. ☐ A change of power of attorney and/or address letter.
19. ☐ A computer-readable form of the sequence listing in accordance with PCT Rule 13ter.2 and 35 U.S.C. 1.821 - 1.825.
20. ☐ A second copy of the published international application under 35 U.S.C. 154(d)(4).
21. ☐ A second copy of the English language translation of the international application under 35 U.S.C. 154(d)(4).
22. ☐ Certificate of Mailing by Express Mail
23. ☒ Other items or information:

Notice of Consideration of Documents Cited in International Search Report

Notice of Priority, PCT/IB/304, PCT/IB/308

U.S. APPLICATION NO. (IF KNOWN, SEE 37 CFR 1.53) 09/1926163		INTERNATIONAL APPLICATION NO. PCT/JP00/01608		ATTORNEY'S DOCKET NUMBER 213930US0PCT	
--	--	---	--	---	--

24. The following fees are submitted:				CALCULATIONS PTO USE ONLY	
BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)) :					
<input type="checkbox"/> Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO				\$1000.00	
<input checked="" type="checkbox"/> International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO				\$860.00	
<input type="checkbox"/> International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO				\$710.00	
<input type="checkbox"/> International preliminary examination fee (37 CFR 1.482) paid to USPTO but all claims did not satisfy provisions of PCT Article 33(1)-(4)				\$690.00	
<input type="checkbox"/> International preliminary examination fee (37 CFR 1.482) paid to USPTO and all claims satisfied provisions of PCT Article 33(1)-(4)				\$100.00	
ENTER APPROPRIATE BASIC FEE AMOUNT =				\$860.00	
Surcharge of \$130.00 for furnishing the oath or declaration later than months from the earliest claimed priority date (37 CFR 1.492 (e)).				\$130.00	
CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE		
Total claims	- 20 =	0	x \$18.00	\$0.00	
Independent claims	- 3 =	0	x \$80.00	\$0.00	
Multiple Dependent Claims (check if applicable).				\$0.00	
TOTAL OF ABOVE CALCULATIONS =				\$990.00	
<input type="checkbox"/> Applicant claims small entity status. (See 37 CFR 1.27). The fees indicated above are reduced by 1/2.				\$0.00	
SUBTOTAL =				\$990.00	
Processing fee of \$130.00 for furnishing the English translation later than months from the earliest claimed priority date (37 CFR 1.492 (f)).				\$130.00	
TOTAL NATIONAL FEE =				\$1,120.00	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31) (check if applicable).				\$0.00	
TOTAL FEES ENCLOSED =				\$1,120.00	
				Amount to be: refunded	\$
				charged	\$

a. ☒ A check in the amount of **\$1,120.00** to cover the above fees is enclosed.


b. ☐ Please charge my Deposit Account No. _____ in the amount of _____ to cover the above fees. A duplicate copy of this sheet is enclosed.

c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. **15-0030**. A duplicate copy of this sheet is enclosed.

d. ☐ Fees are to be charged to a credit card. **WARNING:** Information on this form may become public. **Credit card information should not be included on this form.** Provide credit card information and authorization on PTO-2038.

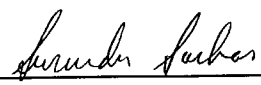
NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:



22850

Surinder Sachar
Registration No. 34,423


 SIGNATURE
Norman F. Oblon
 NAME
24,618
 REGISTRATION NUMBER
 Sept. 17 2001
 DATE

213930US0PCT



09/02/01 16:23:10
Rec'd PCT/PTO 28 MAY 2002

IN THE UNITED STATES PATENT & TRADEMARK OFFICE

IN RE APPLICATION OF :
Takashi SHIBATA, et al. : GROUP ART UNIT:
SERIAL NO: 09/926,163 :
FILED: September 17, 2001 : EXAMINER:
FOR: SORBITOL DEHYDROGENASE, GENE
ENCODING THE SAME AND USE THEREOF

RESPONSE AND STATEMENT

ASSISTANT COMMISSIONER FOR PATENTS
WASHINGTON, D.C. 20231

SIR:

Responsive to the Official Correspondence dated April 24, 2002, Applicants submit herewith a substitute computer-readable Sequence Listing. The paper-copy substitute Sequence Listing was timely filed on March 5, 2002, along with a corresponding computer-readable Sequence Listing.

REMARKS

Claims 1-48 are active in the present application. Favorable consideration is respectfully requested.

Applicants have now submitted a substitute computer-readable Sequence Listing. Contents of the paper copy of the Substitute Sequence Listing filed on March 5, 2002, and the substitute computer-readable Sequence Listing filed herewith are identical. Support for all

the sequences listed in the substitute computer-readable Sequence Listing can be found in the present application. No new matter is introduced by the submission of the substitute computer-readable Sequence Listing.

Applicants submit that this application is in condition for allowance. Early notice to this effect is earnestly solicited.

Respectfully submitted,

OBLON, SPIVAK, McCLELLAND,
MAIER & NEUSTADT, P.C.

Handwritten signature of Norman F. Oblon, with the number #41,253 written next to it.

Norman F. Oblon
Attorney of Record
Registration No. 24,618

Daniel J. Pereira, Ph.D.
Registration No. 45,518

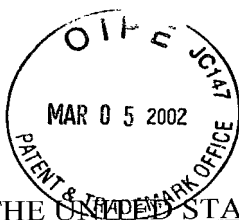


22850

(703) 413-3000
Fax #: (703) 413-2220
NFO/DJPER/TWB:sjh
I:\atty\Twb\213930-pr.wpd

09/26/2001 15:31:22

213930US-0-PCT



IN THE UNITED STATES PATENT & TRADEMARK OFFICE

IN RE APPLICATION OF:

:

Takashi SHIBATA, et al.

:

SERIAL NO: 09/926,163

: ATTN: APPLICATION BRANCH

FILED: September 17, 2001

:

FOR: SORBITOL DEHYDROGENASE, GENE
ENCODING THE SAME AND USE THEREOF

PRELIMINARY AMENDMENT

ASSISTANT COMMISSIONER FOR PATENTS
WASHINGTON, D.C. 20231

SIR:

Applicants submit herewith a substitute Sequence Listing and a corresponding computer-readable Sequence Listing. Prior to examination on the merits, please amend the above-identified application as follows.

IN THE SPECIFICATION

Please amend the specification as follows:

Page 66 (Abstract), after the last line, beginning on the next page, please delete the Sequence Listing at pages insert the substitute Sequence Listing attached hereto.

REMARKS

Claims 1-48 are active in the present application.

Applicants have now submitted a substitute Sequence Listing and a corresponding computer-readable Sequence Listing. Contents of the paper copy of the substitute Sequence

Listing and the computer-readable Sequence Listing are identical. Support for all the sequences listed in the substitute Sequence Listing can be found in the present application. No new matter is introduced by the submission of the substitute Sequence Listing and the computer-readable Sequence Listing.

Applicants submit that this application is in condition for allowance. Early notice to this effect is earnestly solicited.

Respectfully submitted,

OBLON, SPIVAK, McCLELLAND,
MAIER & NEUSTADT, P.C.



Norman F. Oblon
Attorney of Record
Registration No. 24,618

Daniel J. Pereira, Ph.D.
Registration No. 45,518

(703) 413-3000
Fax #: (703) 413-2220
I:\atty\Twb\213930USOPCT-PR.wpd
NFO/DJP/TWB/aps

09/926163
PTO/PCT Rec'd 21 DEC 2001

213930US-0PCT

IN THE UNITED STATES PATENT & TRADEMARK OFFICE

IN RE APPLICATION OF: :
TAKASHI SHIBATA ET AL. :
SERIAL NO: 09/926,163 : ATTN: APPLICATION BRANCH
FILED: SEPTEMBER 17, 2001 :
FOR: SORBITOL DEHYDROGENASE,
GENE ENCODING THE SAME AND
USE THEREOF

PRELIMINARY AMENDMENT

ASSISTANT COMMISSIONER FOR PATENTS
WASHINGTON, D.C. 20231

SIR:

Prior to examination on the merits, please amend the above-identified application as follows.

IN THE SPECIFICATION

Please amend the specification as shown in the attached marked-up copy to read as follows:

Page 4, beginning at line 22 and continuing to page 5, line 2, delete the paragraph and replace it with the following paragraph:

(5) An SLDH which is the following protein (a) or (b):

(a) a protein consisting of an amino acid sequence depicted in Sequence Listing SEQ

ID NO:2

(b) a protein consisting of the same amino acid sequence as

(a) above, except that one to several amino acids are deleted, substituted, inserted, added or modified, which catalyzes a reaction converting D-sorbitol to L-sorbose.

Page 5, lines 5-10, delete the paragraph and replace it with the following paragraph:

(7) The DNA of the above-mentioned (6), which is (a) or (b) of the following:

(a) a DNA having a base sequence of base numbers 537 - 1991 of the base sequence depicted in Sequence Listing SEQ ID NO:1

(b) a DNA capable of hybridizing to the base sequence of the above-mentioned (a) under stringent conditions.

Page 5, lines 13-16, delete the paragraph and replace it with the following paragraph:

(9) A gene encoding a protein having an SLDH activity, which is a DNA capable of hybridizing a DNA having a base sequence of base numbers 537 - 1991 of the base sequence depicted in Sequence Listing SEQ ID NO:1 and a partial DNA thereof.

Page 5, beginning at line 20 and continuing to page 6, line 1, delete the paragraph and replace it with the following paragraph:

(11) A promoter gene comprising the DNA of the following (a) or (b)

(a) a DNA having a base sequence of base numbers 1 - 536 of the base sequence depicted in Sequence Listing SEQ ID NO:1

(b) a DNA having a base sequence of the above-mentioned ~(a) wherein one to several bases is (are) deleted, substituted, inserted, added or modified, which DNA shows a promoter activity at least in one microorganism.

Page 8, beginning at line 21 and continuing to page 9, line 21, delete the paragraph and replace it with the following paragraph:

The SLDH of the present invention is not particularly limited as regards the derivation as long as it shows the above-mentioned characteristics. It may be derived from a naturally occurring organism, a spontaneous or artificial mutant, or a transformant which is obtained by introducing a heterologous (i.e. foreign) SLDH gene. Preferably, SLDH derived

from acetic acid bacteria, particularly bacteria belonging to the genus *Gluconobacter*, more preferably *Gluconobacter oxydans*, particularly the strain *Gluconobacter oxydans* 6624 (FERM BP-4415; International Patent Publication No. W095/23220) are exemplified. In another preferable mode, the SLDH of the present invention is an SLDH derived from the same gene as is the SLDH derived from the strain *G. oxydans* 6624 in its molecular evolution. As used herein, by the "derived from the same gene ... in its molecular evolution" is meant an SLDH reasonably concluded to have evolved from the same gene as has an SLDH derived from strain *G. oxydans* 6624 in its molecular evolution, as a result of the analyses of DNA sequence, physiological role and the like, and their DNA sequences show high homology. These SLDHs preferably have not less than 60%, most preferably not less than 80%, homology in the DNA sequence with an SLDH derived from the strain *G. oxydans* 6624. These genes can be cloned based on the DNA sequence depicted in Sequence Listing SEQ ID NO:1 and using a suitable primer according to the PCR method or using a suitable probe according to the hybridization method, as detailed later.

Page 9, beginning at line 22 and continuing to page 10, line 8, delete the paragraph and replace it with the following paragraph:

In a more preferable mode, the SLDH of the present invention is a protein having an amino acid sequence depicted in Sequence Listing SEQ ID NO:2 or a protein having an amino acid sequence having the amino acid sequence comprising one to several amino acids deleted, substituted, inserted, added or modified, as long as the SLDH activity is not impaired.

Page 11, lines 11-16, delete the paragraph and replace it with the following paragraph:

Production of the SLDH of the present invention by chemical synthesis includes the steps of, for example, synthesizing, based on the amino acid sequence depicted in Sequence Listing SEQ ID NO:2, the entirety or a part of each sequence using peptide synthesizer, and renaturing the obtained polypeptide under suitable renaturation conditions.

Page 17, lines 9-26, delete the paragraph and replace it with the following paragraph:

A DNA encoding the SLDH of the present invention preferably encodes an amino acid sequence depicted in v. Sequence Listing SEQ ID NO:2, or an amino acid sequence wherein, in the above-mentioned amino acid sequence, 1 to several amino acids are deleted, substituted, inserted or added (provided that a protein consisting of the mutated amino acid sequence can catalyze the reaction to convert D-sorbitol to L-sorbose). More preferably, a DNA encoding the SLDH of the present invention is a DNA substantially consisting of a base sequence having a base number 537 - 1991 of the base sequence depicted in Sequence Listing SEQ ID NO:1. As used herein, by the "DNA substantially consisting of" is meant a DNA consisting of this specific base sequence and a DNA consisting of a base sequence capable of hybridizing to the DNA consisting of this specific base sequence under stringent conditions, and encoding a protein having similar physicochemical properties as the protein encoded by the DNA consisting of this specific base sequence.

Page 18, lines 6-14, delete the paragraph and replace it with the following paragraph:

The DNA of the present invention may be a DNA obtained from a genomic DNA as mentioned above, or a cDNA obtained from mRNA, or DNA chemically synthesized based on a base sequence having a base number 537 - 1991 from the base sequence depicted in Sequence Listing SEQ ID NO: 1.

Page 18, lines 15-19, delete the paragraph and replace it with the following paragraph:

The DNA of the present invention may be a DNA obtained from a genomic DNA as mentioned above, or a cDNA obtained from mRNA, or DNA chemically synthesized based on a base sequence having a base number 537 - 1991 from the base sequence depicted in Sequence Listing SEQ ID NO:1.

Page 18, beginning at line 20 and continuing to page 19, line 8, delete the paragraph and replace it with the following paragraph:

Page 35, lines 16-26, delete the paragraph and replace it with the following paragraph:

base sequence of about 4 kb Hind IIIHind III fragment was determined (Sequence Listing SEQ ID NO:1.

Page 48, after the last line beginning on a new page, please replace the original Sequence Listing with the substitute Sequence Listing attached hereto.

IN THE CLAIMS

Please amend the claims as shown on the marked-up copy following this amendment to read as follows.

6. (Amended) A DNA encoding the sorbitol dehydrogenase as claimed in claim 1.
8. (Amended) The DNA of claim 6, which is derived from bacteria belonging to the genus *Gluconobacter*.
12. (Amended) A recombinant vector comprising a DNA as claimed in claim 6.
13. (Amended) An expression vector comprising a DNA as claimed in claim 6.
15. (Amended) A transformant obtained by transforming a host cell with an expression vector of claim 13.
17. (Amended) The transformant of claim 15, which is capable of converting D-sorbitol to 2-keto-L-gluconic acid.
18. (Amended) A method for producing a protein having a sorbitol dehydrogenase activity, which method comprises culturing a host cell transformed with an expression vector of claim 13 in a medium and harvesting the sorbitol dehydrogenase of claim 1 or the protein of claim 10 from the obtained culture.
22. (Amended) A method for producing L-ascorbic acid or an alkali metal salt thereof or an alkaline earth metal salt thereof, which method comprises converting 2-keto-L-

gluconic acid obtained by the method of claim 20 to L-ascorbic acid or an alkali metal salt thereof or an alkaline earth metal salt thereof.

Please add new Claims 23-48.

23. (New) A DNA encoding the sorbitol dehydrogenase as claimed in claim 5.

24. (New) The DNA of claim 23, which is (a) or (b) in the following:

(a) a DNA having a base sequence of base numbers 537 - 1991 of the base sequence depicted in Sequence Listing SEQ ID NO:1

(b) a DNA capable of hybridizing to the base sequence of the above-mentioned (a) under stringent conditions.

25. (New) The DNA of claim 23, which is derived from bacteria belonging to the genus *Gluconobacter*.

26. (New) A recombinant vector comprising a DNA as claimed in claim 23.

27. (New) A recombinant vector comprising a DNA as claimed in claim 9.

28. (New) An expression vector comprising a DNA as claimed in claim 23.

29. (New) An expression vector comprising a DNA as claimed in claim 9.

30. (New) The expression vector of claim 28 further comprising a DNA encoding sorbose dehydrogenase and/or a DNA encoding sorbosone dehydrogenase.

31. (New) The expression vector of claim 29 further comprising a DNA encoding sorbose dehydrogenase and/or a DNA encoding sorbosone dehydrogenase.

32. (New) A transformant obtained by transforming a host cell with an expression vector of claim 28.

33. (New) A transformant obtained by transforming a host cell with an expression vector of claim 29.

34. (New) The transformant of claim 32, which belongs to a genus selected from the group consisting of *Escherichia coli*, the genus *Pseudomonas*, the genus *Gluconobacter*, the genus *Acetobacter* and the genus *Pseudogluconobacter*.

35. (New) The transformant of claim 33, which belongs to a genus selected from the group consisting of *Escherichia coli*, the genus *Pseudomonas*, the genus *Gluconobacter*, the genus *Acetobacter* and the genus *Pseudogluconobacter*.

36. (New) The transformant of claim 32, which is capable of converting D-sorbitol to 2-keto-L-gluconic acid.

37. (New) The transformant of claim 33, which is capable of converting D-sorbitol to 2-keto-L-gluconic acid.

38. (New) A method for producing a protein having a sorbitol dehydrogenase activity, which method comprises culturing a host cell transformed with an expression vector of claim 13 in a medium harvesting the sorbitol dehydrogenase having the following properties

(a) action: catalyzes the reaction converting D-sorbitol to L-sorbose

(b) molecular weight: about 54 kDa

(c) coenzyme: NAD(P)⁺ dependent

(d) substrate specificity: specifically oxidizes sorbitol, mannitol and arbutol, but does not act on xylitol, ribitol, inositol or glycerol, or

a protein derived from the genus *Gluconobacter*, which is encoded by a gene encoding a protein having a sorbitol dehydrogenase activity, which is a DNA capable of hybridizing a DNA having a base sequence of base numbers 537 - 1991 of the base sequence depicted in Sequence Listing SEQ ID NO:1 and a partial DNA thereof, and which has sorbitol dehydrogenase activity, from the obtained culture.

39. (New) A method for producing a protein having a sorbitol dehydrogenase activity, which method comprises culturing a host cell transformed with an expression vector of claim 28 in a medium and harvesting the sorbitol dehydrogenase having the following properties

(a) action: catalyzes the reaction converting D-sorbitol to L-sorbose

(b) molecular weight: about 54 kDa

(c) coenzyme: NAD(P)⁺ dependent

(d) substrate specificity: specifically oxidizes sorbitol, mannitol and arbutol, but does not act on xylitol, ribitol, inositol or glycerol, or

a protein derived from the genus *Gluconobacter*, which is encoded by a gene encoding a protein having a sorbitol dehydrogenase activity, which is a DNA capable of hybridizing a DNA having a base sequence of base numbers 537 - 1991 of the base sequence depicted in Sequence Listing SEQ ID NO:2 and a partial DNA thereof, and which has sorbitol dehydrogenase activity, from the obtained culture.

40. (New) A method for producing a protein having a sorbitol dehydrogenase activity, which method comprises culturing a host cell transformed with an expression vector of claim 29 in a medium and harvesting the sorbitol dehydrogenase having the following properties

(a) action: catalyzes the reaction converting D-sorbitol to L-sorbose

(b) molecular weight: about 54 kDa

(c) coenzyme: NAD(P)⁺ dependent

(d) substrate specificity: specifically oxidizes sorbitol, mannitol and arbutol, but does not act on xylitol, ribitol, inositol or glycerol, or

41. (New) A method for producing an L-sorbose, which method comprises culturing a host cell transformed with an expression vector of claim 28 in a medium and bringing D-sorbitol into contact with the obtained culture or a treated product thereof.

42. (New) A method for producing an L-sorbose, which method comprises culturing a host cell transformed with an expression vector of claim 29 in a medium and bringing D-sorbitol into contact with the obtained culture or a treated product thereof.

43. (New) A method for producing 2-keto-L-gluconic acid, which method comprises culturing a host cell transformed with an expression vector containing a DNA encoding sorbose dehydrogenase and a DNA encoding corbosone dehydrogenase in a medium and bringing the L-sorbose obtained according to the method of claim 41 into contact with the obtained culture or a treated product thereof.

44. (New) A method for producing 2-keto-L-gluconic acid, which method comprises culturing a host cell transformed with an expression vector containing a DNA encoding sorbose dehydrogenase and a DNA encoding corbosone dehydrogenase in a medium and bringing the L-sorbose obtained according to the method of claim 42 into contact with the obtained culture or a treated product thereof.

45. (New) A method for producing 2-keto-L-gluconic acid, which method comprises culturing the transformant of claim 36 in a medium and bringing D-sorbitol into contact with the obtained culture or a treated product thereof.

46. (New) A method for producing 2-keto-L-gluconic acid, which method comprises culturing the transformant of claim 32 in a medium and bringing D-sorbitol into contact with the obtained culture or a treated product thereof.

47. (New) A method for producing L-ascorbic acid or an alkali metal salt thereof or an alkaline earth metal salt thereof, which method comprises converting 2-keto-L-gluconic acid obtained by the method of claim 43 to L-ascorbic acid or an alkali metal salt thereof or an alkaline earth metal salt thereof.

48. (New) A method for producing L-ascorbic acid or an alkali metal salt thereof or an alkaline earth metal salt thereof, which method comprises converting 2-keto-L-gluconic acid obtained by the method of claim 44 to L-ascorbic acid or an alkali metal salt thereof or an alkaline earth metal salt thereof.

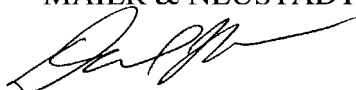
REMARKS

Claims 1-48 are active in the present application. Claims 6, 8, 12-13, 15, 17-18 and 22 have been amended to remove multiple dependencies. Claims 23-48 are new claims. Support for the new claims is found in the original claims. The specification is amended to include a substitute Sequence Listing and to change the Sequence Identifiers (SEQ ID NO:) to correspond to the substitute Sequence Listing.

Applicants submit that the sequence information recorded in the corresponding computer-readable Sequence Listing is identical to the paper copy of the substitute Sequence Listing. Support for all of the sequences listed in the substitute Sequence Listing is found in the present application as originally filed. No new matter is believed to have been introduced by the submission of the substitute Sequence Listing and the foregoing amendments. An action on the merits and allowance of claims is solicited.

Respectfully submitted,

OBLON, SPIVAK, McCLELLAND,
MAIER & NEUSTADT, P.C.



Norman F. Oblon
Attorney of Record
Registration No. 24,618

Daniel J. Pereira, Ph.D.
Registration No. 45,518



22850

(703) 413-3000
NFO/DJP/smi

H:\213930US-pr.wpd

213930US-0PCT

Marked-Up Copy

Serial No:

09/926,163

Amendment Filed on:

12-21-01IN THE SPECIFICATION

Please amend the specification as shown in the attached marked-up copy to read as follows:

Page 4, beginning at line 22 and continuing to page 5, line 2, delete the paragraph and replace it with the following paragraph:

(5) An SLDH which is the following protein (a) or (b):

(a) a protein consisting of an amino acid sequence depicted in Sequence Listing SEQ

ID NO:[1]2

(b) a protein consisting of the same amino acid sequence as

(a) above, except that one to several amino acids are deleted, substituted, inserted, added or modified, which catalyzes a reaction converting D-sorbitol to L-sorbose.

Page 5, lines 5-10, delete the paragraph and replace it with the following paragraph:

(7) The DNA of the above-mentioned (6), which is (a) or (b) of the following:

(a) a DNA having a base sequence of base numbers 537 - 1991 of the base sequence depicted in Sequence Listing SEQ ID NO:[2]1

(b) a DNA capable of hybridizing to the base sequence of the above-mentioned (a) under stringent conditions.

Page 5, lines 13-16, delete the paragraph and replace it with the following paragraph:

(9) A gene encoding a protein having an SLDH activity, which is a DNA capable of hybridizing a DNA having a base sequence of base numbers 537 - 1991 of the base sequence depicted in Sequence Listing SEQ ID NO:[2]1 and a partial DNA thereof.

Page 5, beginning at line 20 and continuing to page 6, line 1, delete the paragraph and replace it with the following paragraph:

(11) A promoter gene comprising the DNA of the following (a) or (b)

(a) a DNA having a base sequence of base numbers 1 - 536 of the base sequence depicted in Sequence Listing SEQ ID NO:[2]1

(b) a DNA having a base sequence of the above-mentioned ~(a) wherein one to several bases is (are) deleted, substituted, inserted, added or modified, which DNA shows a promoter activity at least in one microorganism.

Page 8, beginning at line 21 and continuing to page 9, line 21, delete the paragraph and replace it with the following paragraph:

The SLDH of the present invention is not particularly limited as regards the derivation as long as it shows the above-mentioned characteristics. It may be derived from a naturally occurring organism, a spontaneous or artificial mutant, or a transformant which is obtained by introducing a heterologous (i.e. foreign) SLDH gene. Preferably, SLDH derived from acetic acid bacteria, particularly bacteria belonging to the genus *Gluconobacter*, more preferably *Gluconobacter oxydans*, particularly the strain *Gluconobacter oxydans* 6624 (FERM BP-4415; International Patent Publication No. W095/23220) are exemplified. In another preferable mode, the SLDH of the present invention is an SLDH derived from the same gene as is the SLDH derived from the strain *G. oxydans* 6624 in its molecular evolution. As used herein, by the "derived from the same gene ... in its molecular evolution" is meant an SLDH reasonably concluded to have evolved from the same gene as has an

SLDH derived from strain *G. oxydans* 6624 in its molecular evolution, as a result of the analyses of DNA sequence, physiological role and the like, and their DNA sequences show high homology. These SLDHs preferably have not less than 60%, most preferably not less than 80%, homology in the DNA sequence with an SLDH derived from the strain *G. oxydans* 6624. These genes can be cloned based on the DNA sequence depicted in Sequence Listing SEQ ID NO:[2]1 and using a suitable primer according to the PCR method or using a suitable probe according to the hybridization method, as detailed later.

Page 9, beginning at line 22 and continuing to page 10, line 8, delete the paragraph and replace it with the following paragraph:

In a more preferable mode, the SLDH of the present invention is a protein having an amino acid sequence depicted in Sequence Listing SEQ ID NO:[1]2 or a protein having an amino acid sequence having the amino acid sequence comprising one to several amino acids deleted, substituted, inserted, added or modified, as long as the SLDH activity is not impaired.

Page 11, lines 11-16, delete the paragraph and replace it with the following paragraph:

Production of the SLDH of the present invention by chemical synthesis includes the steps of, for example, synthesizing, based on the amino acid sequence depicted in Sequence Listing SEQ ID NO:[1]2, the entirety or a part of each sequence using peptide synthesizer, and renaturing the obtained polypeptide under suitable renaturation conditions.

Page 17, lines 9-26, delete the paragraph and replace it with the following paragraph:

A DNA encoding the SLDH of the present invention preferably encodes an amino acid sequence depicted in v. Sequence Listing SEQ ID NO:[1]2, or an amino acid sequence wherein, in the above-mentioned amino acid sequence, 1 to several amino acids are deleted,

substituted, inserted or added (provided that a protein consisting of the mutated amino acid sequence can catalyze the reaction to convert D-sorbitol to L-sorbose). More preferably, a DNA encoding the SLDH of the present invention is a DNA substantially consisting of a base sequence having a base number 537 - 1991 of the base sequence depicted in Sequence Listing SEQ ID NO:[2]1. As used herein, by the "DNA substantially consisting of" is meant a DNA consisting of this specific base sequence and a DNA consisting of a base sequence capable of hybridizing to the DNA consisting of this specific base sequence under stringent conditions, and encoding a protein having similar physicochemical properties as the protein encoded by the DNA consisting of this specific base sequence.

Page 18, lines 6-14, delete the paragraph and replace it with the following paragraph:

The DNA of the present invention may be a DNA obtained from a genomic DNA as mentioned above, or a cDNA obtained from mRNA, or DNA chemically synthesized based on a base sequence having a base number 537 - 1991 from the base sequence depicted in Sequence Listing SEQ ID NO:[2] 1.

Page 18, lines 15-19, delete the paragraph and replace it with the following paragraph:

The DNA of the present invention may be a DNA obtained from a genomic DNA as mentioned above, or a cDNA obtained from mRNA, or DNA chemically synthesized based on a base sequence having a base number 537 - 1991 from the base sequence depicted in Sequence Listing SEQ ID NO[2]1.

Page 18, beginning at line 20 and continuing to page 19, line 8, delete the paragraph and replace it with the following paragraph:

Using plasmids pUCP19-HC, pUC18-S1, pUC18-ES and pUC18E1 as templates and using universal primer and reverse primer (New England Labs.), which were M13 sequencing primers, first sequencing was performed. The sample was fluorescent labeled with BigDye Terminator Cycle Sequencing kit (Applied Biosystems) and analyzed with ABI PRISM 310 Genetic Analyzer (Applied Biosystems). The following 11 kinds of primers were synthesized and using pUCP19-HC as a template sequencing was performed, whereby the base sequence of about 4 kb Hind IIIHind III fragment was determined (Sequence Listing SEQ ID NO:[2]1).

-17-

[illegible]

2

5] as claimed in claim 1.

to the genus *Gluconobacter*.

claimed in claim 6.

claimed in claim 6.

expression vector of claim 13 [or 14].

D-sorbitol to 2-keto-L-gluconic acid.

having the following properties

(a) action: catalyzes the reaction converting D-sorbitol to L-sorbose

(b) molecular weight: about 54 kDa

(c) coenzyme: NAD(P)^+ dependent

not act on xylitol, ribitol, inositol or glycerol, or [the protein of claim 10] a protein derived

22. (Amended) A method for producing L-ascorbic acid or an alkali metal salt thereof or an alkaline earth metal salt thereof, which method comprises converting 2-keto-L-gluconic acid obtained by the method of claim 20 [or 21] to L-ascorbic acid or an alkali metal salt thereof or an alkaline earth metal salt thereof.

Claims 23-48 (New).--

09/926163
PTO/PCT Rec'd 21 DEC 2001

SEQUENCE LISTING

<110> SHIBATA, Takashi
ICHIKAWA, Chiyo
MATSUURA, Mitsutaka
NOGUCHI, Yuji
SAITO, Yoshimasa
YAMASHITA, Michio
TAKATA, Yoko

<120> SORBITOL DEHYDROGENASE, GENE ENCODING THE SAME AND USE THEREOF

<130> 213930USOPCT

<140> 09/926,163

<141> 2001-09-17

<150> PCT/JP00/01608

<151> 2000-03-16

<150> JP11/72810

<151> 1999-03-17

<150> JP11/224679

<151> 1999-08-06

<160> 20

<170> PatentIn version 3.1

<210> 1

<211> 4115

<212> DNA

<213> Gluconobacter oxydans

<220>

<221> CDS

<222> (537)..(1994)

<223>

<400> 1

aagcttgc	at	gcctgc	aggt	cgactc	taga	ggatcc	ggtt	ttggc	agcg	tc	cctaga	tt	60
gatgcg	gcgt	ctgttg	accg	acatga	tgct	ggtggc	acgt	gccatt	gcga	cggggc	gtgc	120	
gaccgg	gaac	acaggc	ctgc	tgcctt	tgtg	caaggg	gctg	agtc	atgc	gcgc	tgcgtg	ggct	180
ggcac	atagt	tgcga	agag	agttgc	gcgc	aaagc	agaac	cagcat	gaac	agc	agtcc	ga	240
agacg	aggaa	atcctc	ggcc	tcctac	cgcg	attgga	agag	cagacc	cgtc	ctga	gatgc	gc	300
ttttgt	gatg	tcctgt	tcc	gcgagg	atct	cgaac	gggt	gttggg	gtgc	tcatgc	gttc	360	
tgatgc	gagt	gccgca	aaaag	gtctct	gaac	aggac	gtccc	gcggag	ggca	gtc	agagg	tc	420
gaaatg	gctc	ctgttg	aaaac	cgtcatt	cgg	ttttt	acgtt	gtttc	ggggc	tatga	tggca	480	
catgccc	ggc	cttgtc	ggtc	cccgtc	agcg	accggc	ccga	aaccac	ggag	aattcc	atg	539	
										Met			
										1			

att	acg	cgc	gaa	acc	ctt	aag	tct	ctt	cct	gcc	aat	gtc	cag	gct	ccc	587
Ile	Thr	Arg	Glu	Thr	Leu	Lys	Ser	Leu	Pro	Ala	Asn	Val	Gln	Ala	Pro	
			5						10				15			

ccc	tat	gac	atc	gac	ggg	atc	aag	cct	ggg	atc	gtg	cat	ttc	ggc	gta	635
Pro	Tyr	Asp	Ile	Asp	Gly	Ile	Lys	Pro	Gly	Ile	Val	His	Phe	Gly	Val	

	20					25					30					
ggt Gly	aac Asn	ttt Phe	ttt Phe	cga Arg	gcc Ala	cat His	gag Glu	gcg Ala	ttc Phe	tac Tyr	gtc Val	gag Glu	cag Gln	att Ile	ctt Leu	683
	35					40					45					
gaa Glu	cac His	gct Ala	ccg Pro	gac Asp	tgg Trp	gcg Ala	att Ile	gtt Val	ggc Gly	gtt Val	ggc Gly	ctg Leu	acg Thr	ggc Gly	agt Ser	731
50					55					60					65	
gac Asp	cgt Arg	tca Ser	aag Lys	aaa Lys	aaa Lys	gcc Ala	gag Glu	gaa Glu	ttc Phe	aag Lys	gcc Ala	cag Gln	gac Asp	tgc Cys	ctg Leu	779
				70					75					80		
tat Tyr	tcc Ser	ctg Leu	acc Thr	gag Glu	acg Thr	gct Ala	ccg Pro	tcc Ser	ggc Gly	aag Lys	agc Ser	acg Thr	gtg Val	cgc Arg	gtc Val	827
			85					90					95			
atg Met	ggc Gly	gcg Ala	ctg Leu	cgt Arg	gac Asp	tat Tyr	ctg Leu	ctt Leu	gcc Ala	ccg Pro	gcc Ala	gat Asp	ccg Pro	gaa Glu	gcc Ala	875
		100					105					110				
gtg Val	ctg Leu	aag Lys	cat His	ctt Leu	gtt Val	gat Asp	ccg Pro	gcc Ala	atc Ile	cgc Arg	atc Ile	gtt Val	tcc Ser	atg Met	acg Thr	923
	115					120					125					
atc Ile	acg Thr	gaa Glu	ggc Gly	ggc Gly	tac Tyr	aac Asn	atc Ile	aac Asn	gag Glu	acg Thr	acc Thr	ggc Gly	gcg Ala	ttc Phe	gat Asp	971
130					135					140					145	
ctg Leu	gag Glu	aat Asn	gcg Ala	gca Ala	gta Val	aag Lys	gcc Ala	gac Asp	ctc Leu	aag Lys	aac Asn	ccg Pro	gaa Glu	aag Lys	ccg Pro	1019
				150					155					160		
tct Ser	acc Thr	gtt Val	ttc Phe	ggc Gly	tac Tyr	gtg Val	gtc Val	gag Glu	gcc Ala	ctg Leu	cgt Arg	cgt Arg	cgt Arg	tgg Trp	gat Asp	1067
			165					170					175			
gcc Ala	ggc Gly	ggc Gly	aag Lys	gca Ala	ttt Phe	acg Thr	gtc Val	atg Met	tcc Ser	tgt Cys	gat Asp	aac Asn	ctg Leu	cgt Arg	cat His	1115
		180					185					190				
aac Asn	ggc Gly	aat Asn	gtc Val	gcc Ala	cgc Arg	aag Lys	gcc Ala	ttc Phe	ctc Leu	ggc Gly	tat Tyr	gcg Ala	aag Lys	ggc Ala	cgc Arg	1163
	195					200					205					
gat Asp	ccg Pro	gag Glu	ttg Leu	gcg Ala	aag Lys	tgg Trp	att Ile	gag Glu	gaa Glu	aac Asn	gcg Ala	acc Thr	ttc Phe	ccg Pro	aac Asn	1211
210					215					220					225	
gga Gly	atg Met	gtt Val	gat Asp	cgc Arg	atc Ile	acc Thr	ccg Pro	acc Thr	gtt Val	tgc Ser	gcg Ala	gaa Glu	atc Ile	gcc Ala	aag Lys	1259

230								235				240				
aag	ctc	aac	gcg	gcc	agt	ggg	ctg	gat	gac	gac	ctg	ccg	ctg	gtg	gcc	1307
Lys	Leu	Asn	Ala	Ala	Ser	Gly	Leu	Asp	Asp	Asp	Leu	Pro	Leu	Val	Ala	
			245					250					255			
gag	gat	ttc	cat	cag	tgg	gtg	ctg	gaa	gac	cag	ttt	gcg	gat	ggc	cgt	1355
Glu	Asp	Phe	His	Gln	Trp	Val	Leu	Glu	Asp	Gln	Phe	Ala	Asp	Gly	Arg	
		260					265					270				
ccg	ccg	ctt	gaa	aaa	gcc	ggc	gtg	cag	atg	gtc	ggg	gac	gtg	acg	gac	1403
Pro	Pro	Leu	Glu	Lys	Ala	Gly	Val	Gln	Met	Val	Gly	Asp	Val	Thr	Asp	
		275				280					285					
tgg	gag	tac	gtc	aag	atc	cga	atg	ctc	aat	gca	ggg	cat	gtc	atg	ctc	1451
Trp	Glu	Tyr	Val	Lys	Ile	Arg	Met	Leu	Asn	Ala	Gly	His	Val	Met	Leu	
290					295					300					305	
tgc	ttc	cca	ggc	att	ctg	gtc	ggc	tat	gag	aat	gtg	gat	gac	gcc	att	1499
Cys	Phe	Pro	Gly	Ile	Leu	Val	Gly	Tyr	Glu	Asn	Val	Asp	Asp	Ala	Ile	
				310					315					320		
gaa	gac	agc	gaa	ctc	ctt	ggc	aat	ctg	aag	aac	tat	ctc	aac	aag	gat	1547
Glu	Asp	Ser	Glu	Leu	Leu	Gly	Asn	Leu	Lys	Asn	Tyr	Leu	Asn	Lys	Asp	
			325					330					335			
gtc	atc	ccg	acc	ctg	aag	gcg	cct	tca	ggc	atg	acg	ctc	gaa	ggc	tat	1595
Val	Ile	Pro	Thr	Leu	Lys	Ala	Pro	Ser	Gly	Met	Thr	Leu	Glu	Gly	Tyr	
		340					345					350				
cgg	gac	agc	gtc	atc	agc	cgt	ttc	tcc	aac	aag	gcg	atg	tcg	gac	cag	1643
Arg	Asp	Ser	Val	Ile	Ser	Arg	Phe	Ser	Asn	Lys	Ala	Met	Ser	Asp	Gln	
	355					360					365					
acg	ctc	cgg	att	gct	agc	gat	ggc	tgt	tcc	aag	gtt	cag	gtg	ttc	tgg	1691
Thr	Leu	Arg	Ile	Ala	Ser	Asp	Gly	Cys	Ser	Lys	Val	Gln	Val	Phe	Trp	
370					375					380					385	
acg	gaa	acc	gtg	cgt	cgg	gcg	atc	gaa	gac	aag	cgg	gac	ctg	tca	cgt	1739
Thr	Glu	Thr	Val	Arg	Arg	Ala	Ile	Glu	Asp	Lys	Arg	Asp	Leu	Ser	Arg	
				390					395					400		
ata	gcg	ttc	gga	att	gca	tcc	tat	ctc	gaa	atg	ctg	cgt	ggc	cgc	gac	1787
Ile	Ala	Phe	Gly	Ile	Ala	Ser	Tyr	Leu	Glu	Met	Leu	Arg	Gly	Arg	Asp	
			405					410					415			
gag	aag	ggc	ggg	acg	tat	gaa	tcg	tcc	gag	ccg	act	tat	ggc	gac	gcc	1835
Glu	Lys	Gly	Gly	Thr	Tyr	Glu	Ser	Ser	Glu	Pro	Thr	Tyr	Gly	Asp	Ala	
		420					425					430				
gaa	tgg	aag	ttg	gcc	aag	gcg	gac	gac	ttc	gaa	agc	tct	ctg	aag	ctc	1883
Glu	Trp	Lys	Leu	Ala	Lys	Ala	Asp	Asp	Phe	Glu	Ser	Ser	Leu	Lys	Leu	

435				440				445								
ccg gcg ttc gat ggg tgg cgc gat ctg gat acg tcc gaa ctg gat caa	1931															
Pro Ala Phe Asp Gly Trp Arg Asp Leu Asp Thr Ser Glu Leu Asp Gln																
450					455					460					465	
aag gtc atc gtg ctg cgg aag atc atc cgc gaa aag ggc gta aaa gcc	1979															
Lys Val Ile Val Leu Arg Lys Ile Ile Arg Glu Lys Gly Val Lys Ala																
				470					475					480		
gcc atc ccg gcc tga attcggcctt tagggtagcg actgaaacag aaaaccgcgc	2034															
Ala Ile Pro Ala																
485																
tctggaagga ggcgcggtttt ttttatgctc agatctgtcc catcaggaca aggatcacga	2094															
cgaccacgat caggacaagt ccgctggagg gggagcccca tttcgaactg tacggccatg	2154															
acggcagcgc accgagatca ggattacaag aaggatcagt cccatggcac atctctcttg	2214															
ccggttgaga ctggtctgtg ttccgggtgt ctaaaaagtt tccgtagggg cgcgaaagat	2274															
caaagctgtc ggtcgcgctt aatccgggtcc caagccgcac tgatgcgggc cacccggtcc	2334															
tgtgcgcgctt tgcgctctgt ctctgacata ggtttctggg ccagcacgtc cggatgatgt	2394															
tcgcggatca gggtgcgcca gcgcacgcgg atttctgtgt cagttgcgct gcgggtgatg	2454															
ccgagaatac gataggcatc cggctcgttt ccgctggcgg cgcgattgtt gccgctttcg	2514															
gcccgggtccc atgctcctgg cggcaggcca aatgccccgt gaacgcgctg cagaaaatcg	2574															
atttccttcg ggtgaagctc gcggctgggg ccggcatcgg cacgggcat acggaacagt	2634															
gccgtcatga ggttctcaag cggcgccgta ttatcggcat aggcttgcc catttcgcgg	2694															
gcatacatct cgaaatcgtc cgtccggtcg cgggcgcgat cgaacagcat gccgacttcc	2754															
ttggtgttat cgggggggaa ctggaagcag gtcttgaaag cgttgatttc gtgtcggttc	2814															
accggcccgt cgatcttcgc cagcttcgcg cacagggcaa caaggccgat ggcgtaaagc	2874															
tgatctcgctt tgcccagggc cgcagcaatc ttggcagcgc cgaaaaaggc cgcgctgttg	2934															
ggatcgggac ggccattcgc gggaaagcgc tcactccagc cgcccgttga gggcttgagt	2994															
agcgaaccgt tatcggcggc atgccccagc gctgcgcccc tcagtgtctc gaaaggacca	3054															
ccaaccgcga agcccgcgac accaccgaac atcttgcccc agatagccat gtcatcaacc	3114															
tagcacgccc gctcacagcg gcaaattgaca gatcgcaggc taggtgtagg tgctgatgcg	3174															

ccaaccgccc gggcttgccg tgtggtagaa gctaggagtt acgaacttat cgctgtctca 3234
tgcttttgag ggcaggttc ttctgttcgt ttcatgacgg atatttttat gccaccttg 3294
atccagactg ctacttcgat ccctttccgc tctgatgacg aactgatgga tcttttgatc 3354
aagcgtctgc caatgtggct gcagaaagtg ctgaactggg tgcgggaagc ggatcataaa 3414
tgggttcgga ttccggcggg cgtgctgttc atgctggcg gcgttctgtc catcctgcct 3474
gttctgggtc tgtggatgct gccggtcggc gtgatgttgc ttgcgcagga tattccgttc 3534
ttccgtcgcc ttccaggccg cctcttgccg tggatcgaac gtcaacatcc ggattggctg 3594
ggccttcgga cgaaaagcgg cagaagctaa ccgttcgtct ggacgtgttt ctgaagatgt 3654
gtcagtgtcg caaccgcag ggctgaagcc agtggcgct ctggtggctg cgcggcatcg 3714
agagaagcca ccagagacgc aaagctctgc tggcggactg cggccatcg gtccagtata 3774
gcccagaact cgggttcag tgccacggac gtccggtgtc ctgacagaga caggctgcgt 3834
ttgacgagat cactcattcc ggttggttct caaggcgctt caaagcccat tgtgcggttt 3894
cggaaacatc aggggtccga tcaactcagca gctcccgcgc agaagatata agcgacggat 3954
cggccgaagt gccgatcgcg atcaggacag ttacgtacga accggttgcg tccaatccgt 4014
ttgaccggag agccagaaaa aaacgtccgg aatgtcgcat tatccagccg caccagttcg 4074
tcgagttttg gtgcaatcag ctccggcgcg gcctgaagct t 4115

<210> 2

<211> 485

<212> PRT

<213> Gluconobacter oxydans

<400> 2

Met Ile Thr Arg Glu Thr Leu Lys Ser Leu Pro Ala Asn Val Gln Ala
1 5 10 15

Pro Pro Tyr Asp Ile Asp Gly Ile Lys Pro Gly Ile Val His Phe Gly
20 25 30

Leu Glu His Ala Pro Asp Trp Ala Ile Val Gly Val Gly Leu Thr Gly
50 55 60

Ser Asp Arg Ser Lys Lys Lys Ala Glu Glu Phe Lys Ala Gln Asp Cys
65 70 75 80

Leu Tyr Ser Leu Thr Glu Thr Ala Pro Ser Gly Lys Ser Thr Val Arg
85 90 95

Val Met Gly Ala Leu Arg Asp Tyr Leu Leu Ala Pro Ala Asp Pro Glu
100 105 110

Ala Val Leu Lys His Leu Val Asp Pro Ala Ile Arg Ile Val Ser Met
115 120 125

Thr Ile Thr Glu Gly Gly Tyr Asn Ile Asn Glu Thr Thr Gly Ala Phe
130 135 140

Asp Leu Glu Asn Ala Ala Val Lys Ala Asp Leu Lys Asn Pro Glu Lys
145 150 155 160

Pro Ser Thr Val Phe Gly Tyr Val Val Glu Ala Leu Arg Arg Arg Trp
165 170 175

Asp Ala Gly Gly Lys Ala Phe Thr Val Met Ser Cys Asp Asn Leu Arg
180 185 190

His Asn Gly Asn Val Ala Arg Lys Ala Phe Leu Gly Tyr Ala Lys Ala
195 200 205

Arg Asp Pro Glu Leu Ala Lys Trp Ile Glu Glu Asn Ala Thr Phe Pro
210 215 220

Asn Gly Met Val Asp Arg Ile Thr Pro Thr Val Ser Ala Glu Ile Ala
225 230 235 240

[illegible]

Lys Lys Leu Asn Ala Ala Ser Gly Leu Asp Asp Asp Leu Pro Leu Val
245 250 255

Ala Glu Asp Phe His Gln Trp Val Leu Glu Asp Gln Phe Ala Asp Gly
260 265 270

- Arg Pro Pro Leu Glu Lys Ala Gly Val Gln Met Val Gly Asp Val Thr
275 280 285

Asp Trp Glu Tyr Val Lys Ile Arg Met Leu Asn Ala Gly His Val Met
290 295 300

Leu Cys Phe Pro Gly Ile Leu Val Gly Tyr Glu Asn Val Asp Asp Ala
305 310 315 320

Ile Glu Asp Ser Glu Leu Leu Gly Asn Leu Lys Asn Tyr Leu Asn Lys
325 330 335

Asp Val Ile Pro Thr Leu Lys Ala Pro Ser Gly Met Thr Leu Glu Gly
340 345 350

Tyr Arg Asp Ser Val Ile Ser Arg Phe Ser Asn Lys Ala Met Ser Asp
355 360 365

Gln Thr Leu Arg Ile Ala Ser Asp Gly Cys Ser Lys Val Gln Val Phe
370 375 380

Trp Thr Glu Thr Val Arg Arg Ala Ile Glu Asp Lys Arg Asp Leu Ser
385 390 395 400

Arg Ile Ala Phe Gly Ile Ala Ser Tyr Leu Glu Met Leu Arg Gly Arg
405 410 415

Asp Glu Lys Gly Gly Thr Tyr Glu Ser Ser Glu Pro Thr Tyr Gly Asp
420 425 430

Ala Glu Trp Lys Leu Ala Lys Ala Asp Asp Phe Glu Ser Ser Leu Lys
435 440 445

Leu Pro Ala Phe Asp Gly Trp Arg Asp Leu Asp Thr Ser Glu Leu Asp
 450 455 460

Gln Lys Val Ile Val Leu Arg Lys Ile Ile Arg Glu Lys Gly Val Lys
 465 470 475 480

Ala Ala Ile Pro Ala
 485

<210> 3

<211> 16

<212> DNA

<213> Artificial Sequence

<220>

<223> synthetic DNA

<400> 3

gctgctgagt gatccg

16

<210> 4

<211> 17

<212> DNA

<213> Artificial Sequence

<220>

<223> synthetic DNA

<400> 4

gactgctact tcgatcc

17

<210> 5

<211> 16

<212> DNA

<213> Artificial Sequence

<220>

<223> synthetic DNA

<400> 5

cctacaccta gcctgc

16

<210> 6

<211> 16

<212> DNA

<213> Artificial Sequence

<220>

<223> synthetic DNA

<400> 6

cagtgccgtc atgagg

16

<210> 7

<211> 16

<212> DNA

<213> Artificial Sequence

<220>

<223> synthetic DNA

<400> 7

tcctgatctc ggtgcg

16

<210> 8

<211> 16

<212> DNA

<213> Artificial Sequence

<220>

<223> synthetic DNA

<400> 8

gatgcttcag cacggc

16

<210> 9

<211> 16

<212> DNA

<213> Artificial Sequence

<220>

<223> synthetic DNA

<400> 9

gacgatcacg gaaggc

16

<210> 10

<211> 16

<212> DNA

<213> Artificial Sequence

<220>

<223> synthetic DNA

<400> 10

ggttacgtgg tcgacg

16

<210> 11

.<211> 17

<212> DNA

<213> Artificial Sequence

 $\langle 220 \rangle$

<223> synthetic DNA

<400> 11

ctatacctga caggtcc

17

<210> 12

<211> 16

<212> DNA

<213> Artificial Sequence

 $\langle 220 \rangle$

<223> synthetic DNA

<400> 12

gcgcgatctg gatacg

16

<210> 13

<211> 16

<212> DNA

<213> Artificial Sequence

 $\langle 220 \rangle$

<223> synthetic DNA

<400> 13

cgaggatctc gaacgg

16

<210> 14

<211> 18

<212> DNA

<213> Artificial Sequence

<220>

<223> synthetic DNA

<400> 14

cggattgcta gcgatggc

18

<210> 15

<211> 47

<212> DNA

<213> Artificial Sequence

<220>

<223> synthetic DNA

<400> 15

atcgaggatc ctcaatgatg atgatgatga tgggccggga tggcggc

47

<210> 16

<211> 30

<212> DNA

<213> Artificial Sequence

<220>

<223> synthetic DNA

<400> 16

atcgaggatc cattcggcctt ttagggtagc

30

<210> 17

<211> 28

<212> DNA

<213> Artificial Sequence

<220>

<223> synthetic DNA

<400> 17

tagctgagct catgggacag atctgagc

28

<210> 18

<211> 10

<212> PRT

<213> Gluconobacter oxydans

<400> 18

Met	Ile	Thr	Arg	Glu	Thr	Leu	Lys	Ser	Leu
1				5					10

<210> 19

<211> 33

<212> DNA

<213> Artificial Sequence

<220>

<223> synthetic DNA

<400> 19

taggaatatt tctcatgatt acgcgcgaaa ccc

33

<210> 20

<211> 16

<212> DNA

<213> Artificial Sequence

<220>

<223> synthetic DNA

<400> 20

gatgcttcag cacggc

16

PTO/PCT Rec'd 21 DEC 2001

SPECIFICATION

SORBITOL DEHYDROGENASE, GENE ENCODING THE SAME AND USE
THEREOF

TECHNICAL FIELD

5 This invention relates to a novel sorbitol dehydrogenase (in the present invention, sorbitol dehydrogenase means an enzyme capable of catalyzing a reaction for converting D-sorbitol to L-sorbose by oxidation; hereinafter to be referred to as SLDH), a gene encoding the
10 same, a method for producing L-sorbose and 2-keto-L-gulonic acid (hereinafter to be referred to as 2KLGA) by gene manipulation using said gene, and an expression system involved in the production thereof. The present invention further relates to a method for producing L-ascorbic acid or
15 a salt thereof utilizing the 2KLGA obtained by the above-mentioned method.

BACKGROUND ART

L-sorbose is an important intermediate for the synthesis of L-ascorbic acid (vitamin C) by the Reichstein
20 method (see Fig. 1). When D-sorbitol is chemically oxidized, approximately a half of the product becomes D-sorbose, whereas when D-sorbitol is brought into contact with a microorganism having an SLDH activity, only an L-enantiomer is obtained in a yield of about 95%. Therefore, a
25 fermentation method has been conventionally used for converting D-sorbitol to L-sorbose.

production of 2KLGA, and to provide a host microorganism transformed with said gene, particularly a transformant obtained by introducing said gene into a host already having SDH and SNDH activity, or a transformant obtained by
 5 introducing said gene together with SDH gene and SNDH gene. Another object of the present invention is to provide a method for producing L-sorbose or 2KLGA from D-sorbitol using said microorganism, and to provide a method for producing L-ascorbic acid from 2KLGA obtained by this method. It is yet
 10 another object of the present invention to provide a method for producing a recombinant SLDH by culture of a host microorganism transformed with said SLDH gene and a method for producing L-sorbose by an enzyme method using said SLDH.

DISCLOSURE OF THE INVENTION

15 The present inventors have conducted intensive studies in an attempt to solve the above-mentioned problems and succeeded in cloning a DNA containing a coding region of SLDH from a chromosomal DNA library of a strain belonging to the genus *Gluconobacter* having said enzyme activity. As a result
 20 of the sequencing, the DNA was confirmed to contain a novel SLDH gene completely different from the SLDH gene previously isolated by the present inventors. Moreover, the present inventors transformed *Pseudomonas* with an expression vector containing the DNA and succeeded in purifying a recombinant
 25 SLDH from the culture of said recombinant *Pseudomonas*. They have also transformed *Pseudomonas* transformed with an

expression vector containing said DNA, with an expression
vector containing an SDH gene and an SNDH gene and
efficiently converting D-sorbitol to 2KLGA using the culture
of this transformant, which resulted in the completion of the
5 present invention.

Accordingly, the present invention provides the
following.

- (1) An SLDH having the following physicochemical properties:
 - (a) action: catalyzes the reaction converting D-sorbitol to
10 L-sorbose
 - (b) molecular weight: about 54 kDa
 - (c) coenzyme: NAD(P)⁺ dependent
 - (d) substrate specificity: specifically oxidizes sorbitol,
mannitol and arabitol, but does not act on xylitol, ribitol,
15 inositol and glycerol.
- (2) The SLDH of the above-mentioned (1), which is derived
from the strain *Gluconobacter oxydans* G624.
- (3) An SLDH which is originated from the same gene as is the
SLDH of the above-mentioned (2) in its molecular evolution.
- 20 (4) The SLDH of the above-mentioned (3), which is derived
from a bacteria belonging to the genus *Gluconobacter*.
- (5) An SLDH which is the following protein (a) or (b):
 - (a) a protein consisting of an amino acid sequence depicted
in Sequence Listing SEQ ID NO:1
 - 25 (b) a protein consisting of the same amino acid sequence as
(a) above, except that one to several amino acids are

deleted, substituted, inserted, added or modified, which catalyzes a reaction converting D-sorbitol to L-sorbose.

(6) A DNA encoding the SLDH of any of the above-mentioned (1) to (5).

5 (7) The DNA of the above-mentioned (6), which is (a) or (b) of the following:

(a) a DNA having a base sequence of base numbers 537 - 1991 of the base sequence depicted in Sequence Listing SEQ ID NO:2

(b) a DNA capable of hybridizing to the base sequence of the
10 above-mentioned (a) under stringent conditions.

(8) The DNA of the above-mentioned (6) or (7), which is derived from bacteria belonging to the genus *Gluconobacter*.

(9) A gene encoding a protein having an SLDH activity, which is a DNA capable of hybridizing a DNA having a base sequence
15 of base numbers 537 - 1991 of the base sequence depicted in Sequence Listing SEQ ID NO:2 and a partial DNA thereof.

(10) A protein derived from the genus *Gluconobacter*, which is encoded by the gene of the above-mentioned (9) and which has an SLDH activity.

20 (11) A promoter gene comprising the DNA of the following (a) or (b):

(a) a DNA having a base sequence of base numbers 1 - 536 of the base sequence depicted in Sequence Listing SEQ ID NO:2

(b) a DNA having a base sequence of the above-mentioned (a)
25 wherein one to several bases is(are) deleted, substituted, inserted, added or modified, which DNA shows a promoter

expression vector of the above-mentioned (13) in a medium and bringing D-sorbitol into contact with the obtained culture or a treated product thereof.

(20) A method for producing 2-KLGA, which method comprises
5 culturing a host cell transformed with an expression vector containing a DNA encoding an SDH and a DNA encoding an SNDH in a medium and bringing the L-sorbose obtained according to the method of the above-mentioned (19) into contact with the obtained culture or a treated product thereof.

10 (21) A method for producing 2-KLGA, which method comprises culturing the transformant of the above-mentioned (17) in a medium and bringing D-sorbitol into contact with the obtained culture or a treated product thereof.

(22) A method for producing L-ascorbic acid or an alkali
15 metal salt thereof or an alkaline earth metal salt thereof, which method comprises converting 2-KLGA obtained by the method of the above-mentioned (20) or (21) to L-ascorbic acid or an alkali metal salt thereof or an alkaline earth metal salt thereof.

20 The recombinant cell that expresses the SLDH gene of the present invention can be a useful means for the fermentative production of L-sorbose and 2KLGA. Therefore, the present invention is extremely useful for facilitated and large-scale production of L-ascorbic acid.

25 **BRIEF DESCRIPTION OF THE DRAWINGS**

FIG. 1 shows a reaction scheme of the synthesis of L-

derived from acetic acid bacteria, particularly bacteria
belonging to the genus *Gluconobacter*, more preferably
Gluconobacter oxydans, particularly the strain *Gluconobacter*
oxydans G624 (FERM BP-4415; International Patent Publication
5 No. WO95/23220) are exemplified. In another preferable mode,
the SLDH of the present invention is an SLDH derived from the
same gene as is the SLDH derived from the strain *G. oxydans*
G624 in its molecular evolution. As used herein, by the
"derived from the same gene ... in its molecular evolution" is
10 meant an SLDH reasonably concluded to have evolved from the
same gene as has an SLDH derived from strain *G. oxydans* G624
in its molecular evolution, as a result of the analyses of
DNA sequence, physiological role and the like, and their DNA
sequences show high homology. These SLDHs preferably have
15 not less than 60%, most preferably not less than 80%,
homology in the DNA sequence with an SLDH derived from the
strain *G. oxydans* G624. These genes can be cloned based on
the DNA sequence depicted in Sequence Listing SEQ ID NO:2 and
using a suitable primer according to the PCR method or using
20 a suitable probe according to the hybridization method, as
detailed later.

In a more preferable mode, the SLDH of the present
invention is a protein having an amino acid sequence depicted
in Sequence Listing SEQ ID NO:1, or a protein having an amino
25 acid sequence having the amino acid sequence comprising one
to several amino acids deleted, substituted, inserted, added

or modified, as long as the SLDH activity is not impaired.

The SLDH of the present invention can be obtained by appropriately using (1) a method comprising isolating and purifying it from a culture of a cell or tissue as a starting
5 material that produces the enzyme, (2) a method comprising chemical synthesis, (3) a method comprising purifying it from a cell manipulated by gene recombinant technique to express SLDH and the like.

The isolation and purification of SLDH from a naturally
10 occurring SLDH producing cell includes, for example, the following steps. The cell is cultured in a suitable liquid medium, and a fraction having an SLDH activity is separated and recovered from the obtained culture. For example, when the enzyme is localized in cytosol (the SLDH of the present
15 invention being NAD(P)^+ dependent, localization in cytosol is expected), the culture is centrifuged and/or filtrated to recover the cell, and the cell is ruptured by ultrasonication, lysozyme treatment, osmotic pressure shock and the like and centrifuged at about 10,000 - 40,000 rpm to
20 recover a supernatant (soluble fraction). The objective SLDH can be purified from the obtained soluble fraction by appropriately combining separation techniques conventionally used for separation and purification of enzyme proteins. Such separation techniques include, for example, methods
25 utilizing difference in solubility such as salting out, solvent precipitation method and the like, methods utilizing

difference in molecular weight such as dialysis,
 ultrafiltration, gel filtration, non-denatured polyacrylamide
 gel electrophoresis (PAGE), SDS-PAGE and the like, methods
 utilizing charge such as ion exchange chromatography,
 5 hydroxyl apatite chromatography and the like, methods
 utilizing specific affinity such as affinity chromatography
 and the like, methods utilizing hydrophobicity such as
 reverse-phase high performance liquid chromatography and the
 like, and methods utilizing difference in isoelectric point
 10 such as isoelectric focusing and the like.

Production of the SLDH of the present invention by
 chemical synthesis includes the steps of, for example,
 synthesizing, based on the amino acid sequence depicted in
 Sequence Listing SEQ ID NO:1, the entirety or a part of each
 15 sequence using peptide synthesizer, and renaturing the
 obtained polypeptide under suitable renaturation conditions.

The SLDH derived from *G. oxydans* G624, which is one
 mode of the present invention, is an extremely unstable
 enzyme in non-physiological conditions, and may be
 20 deactivated during purification by the above-mentioned
 method. Such enzyme can be quickly purified by affinity
 chromatography utilizing an added/modified sequence having
 affinity for a specific substance, according to the histidine
 tag method, GST method and the like. Therefore, a
 25 particularly preferable method for obtaining the SLDH of the
 present invention includes the steps of, as detailed in the

following, cloning a DNA encoding the enzyme from the DNA of a cell having this enzyme, and adding, to this DNA by gene manipulation, a nucleotide sequence encoding an amino acid sequence capable of adsorbing to a metal ion chelate.

5 An enzyme gene can be generally cloned by the following method. A desired enzyme is purified completely or partially from a cell or tissue that produces the enzyme by the above-mentioned method, and the N terminal amino acid sequence is determined by the Edman method. The enzyme is partially
10 digested by a sequence-specific protease and the amino acid sequence of the obtained oligopeptide is also determined by the Edman method. Oligonucleotides having base sequences corresponding to the amino acid sequences thus determined are synthesized, and using these as primers or probes, a DNA
15 encoding this enzyme is cloned from RNA or DNA prepared from a cell or tissue capable of producing the enzyme, by the PCR method or colony (or plaque) hybridization method.

Alternatively, an antibody against the enzyme is prepared using the entirety or a part of a completely or
20 partially purified enzyme as an antigen by a conventional method, and a DNA encoding the enzyme can be cloned from a cDNA or genomic DNA library prepared from a cell or tissue capable of producing the enzyme, by immunoscreening.

However, in the case of an enzyme that is unstable and
25 whose purification is difficult, such as the above-mentioned SLDH derived from *G. oxydans* G624, the gene of the enzyme can

having an SLDH activity are selected using, for example, a color identification reaction with ketohexose, such as a resorcin-hydrochloric acid reaction (Cohen, *J. Biol. Chem.*, 201, 71, 1953), a resorcin-ferric salt-hydrochloric acid
 5 reaction (Kulka, *Biochem. J.*, 63, 542, 1956) and the like.

The presence of SLDH activity (conversion of D-sorbitol to L-sorbose) in the obtained clone is confirmed by the detection of sorbose in the culture supernatant by, for example, HPLC and the like.

10 Because the DNA insert of cosmid clone is considerably large (35 - 45 kb), a part of a non-SLDH gene region of the insert DNA is desirably removed for downsizing for facilitated subcloning to plasmid. For downsizing of the DNA insert, for example, subcloning to a charomid vector and the
 15 like is employed. Since a charomid vector has a spacer DNA of various lengths, DNA having various lengths smaller than a cosmid vector can be cloned. In the present invention, for example, a charomid vector capable of accommodating an about 10 - 20kb DNA insert is preferably used. The charomid clone
 20 having an SLDH activity can be selected according to the method mentioned above.

The subcloning to a plasmid vector can be done by, for example, applying plural charomid clones obtained as mentioned above to restriction enzyme mapping, downsizing a
 25 DNA insert using a restriction enzyme found to have no restriction site in the SLDH gene, and ligating with a

plasmid vector that underwent a restriction enzyme treatment.

Apart from the above-mentioned strategy, moreover, a DNA encoding the SLDH of the present invention can be directly cloned using the PCR method. That is, PCR is conducted according to a conventional method, using a genomic DNA or cDNA (or mRNA) derived from a cell or tissue having the enzyme activity as a template, and using a pair of oligonucleotides, where an amplification fragment suitably covers the coding region of SLDH, as a primer to amplify a DNA fragment containing the coding region of SLDH. This method is particularly useful for cloning of an SLDH gene having the same origin in the molecular evolution with an SLDH having a known sequence. For example, when an SLDH gene derived from a bacteria, which is speculated to have the same origin in the molecular evolution with an SLDH derived from the strain *G. oxydans* G624, is to be cloned, sense and antisense primers capable of amplifying a DNA fragment having high homology with a DNA fragment containing a base sequence of base number 537 - 1991 from the sequence is constructed based on the DNA sequence depicted in Sequence Listing SEQ ID NO:2 and the PCR method is performed. When the DNA sequence of SLDH having high homology with the objective SLDH is unknown, for example, PCR is performed using some sequences conserved relatively well in the 5' upstream region as sense primers, and some complementary strand sequences conserved relatively well in the 3' downstream region as antisense

primers to clone the SLDH gene. When the upstream and downstream sequences of SLDH are unknown, the annealing temperature needs to be set lower, so that a template DNA and a primer to be used containing some mismatches can still be bound. Therefore, the PCR product may be a mixture of a fragment containing the objective SLDH gene and a non-specific amplification fragment. In this case, the obtained amplification fragment is cloned to a suitable cloning vector (for example, plasmid vector for TA cloning and the like).

When the objective amplification fragment does not contain a promoter region, the obtained amplification fragment is cloned to an expression vector, with which a competent cell, such as *Escherichia coli*, is transformed, and the transformant having an SLDH activity is screened by the aforementioned method.

As a different strategy for the cloning of an SLDH gene having the same origin in the molecular evolution with an SLDH having a known sequence, direct cloning by hybridization method such as Southern method and the like may be employed, wherein a genomic DNA or cDNA (or mRNA) derived from a cell or tissue having an SLDH activity is used as a template and the entirety or a part of a known DNA sequence is used as a probe. The conditions of the hybridization may be an appropriately altered stringency depending on the origin of the DNA. For example, the conditions may be changed based on the degree of closeness in the relation of the microorganism

to be cloned and the like, such as those under which, of the base sequence, only a sequence having about not less than 60% homology forms a hybrid, only a sequence having about not less than 80% homology forms a hybrid, and the like.

5 The base sequence of the DNA insert obtained in the above-mentioned manner can be identified by a known sequencing technique, such as Maxam-Gilbert method, dideoxy termination method and the like.

A DNA encoding the SLDH of the present invention
10 preferably encodes an amino acid sequence depicted in Sequence Listing SEQ ID NO:1, or an amino acid sequence wherein, in the above-mentioned amino acid sequence, 1 to several amino acids are deleted, substituted, inserted or added (provided that a protein consisting of the mutated
15 amino acid sequence can catalyze the reaction to convert D-sorbitol to L-sorbose). More preferably, a DNA encoding the SLDH of the present invention is a DNA substantially consisting of a base sequence having a base number 537 - 1991 of the base sequence depicted in Sequence Listing SEQ ID
20 NO:2. As used herein, by the "DNA substantially consisting of" is meant a DNA consisting of this specific base sequence and a DNA consisting of a base sequence capable of hybridizing to the DNA consisting of this specific base sequence under stringent conditions, and encoding a protein
25 having similar physicochemical properties as the protein encoded by the DNA consisting of this specific base sequence.

The "stringent conditions" here mean those under which a DNA having about not less than 60% homology of base sequence can hybridize. The stringency can be controlled by appropriately changing the temperature, salt concentration and the like of hybridization reaction and washing.

Another DNA of the present invention also encompasses a gene consisting of a base sequence capable of hybridizing to a base sequence having a base number 537 - 1991 from the base sequence depicted in Sequence Listing SEQ ID NO:2 and a partial DNA thereof, and encoding a protein having an SLDH activity. Therefore, a protein having an SLDH activity, which is encoded by this gene, particularly a protein derived from the genus *Gluconobacter*, is also within the scope of the present invention.

The DNA of the present invention may be a DNA obtained from a genomic DNA as mentioned above, or a cDNA obtained from mRNA, or DNA chemically synthesized based on a base sequence having a base number 537 - 1991 from the base sequence depicted in Sequence Listing SEQ ID NO:2.

The DNA encoding SLDH, which is obtained from a genomic DNA with the SLDH activity as an index as mentioned above, contains a promoter gene sequence in the 5' upstream region. This promoter gene preferably has a base sequence having a base number 1 - 536 from the base sequence depicted in Sequence Listing SEQ ID NO:2, or said base sequence wherein one to several amino acids are deleted, substituted,

inserted, added or modified, which is a DNA having a promoter activity in at least one microorganism. As the "microorganism" here, there are preferably exemplified prokaryotes such as bacteria (e.g., *Escherichia coli*, *Bacillus subtilis*, *Pseudomonas*, *Gluconobacter*, *Pseudogluconobacter*, *Acetobacter* and the like) and actinomyces, and certain eucaryotes such as yeast and the like.

The present invention provides a recombinant vector containing a DNA encoding the SLDH of the present invention. The recombinant vector of the present invention is not particularly limited as long as it can replicate/maintain or autonomously proliferate in various host cells of procaryotic and/or eucaryotic cells, and encompasses a plasmid vector, a phage vector and the like. The recombinant vector can be conveniently prepared by inserting a DNA encoding SLDH into a cloning vector or expression vector available in the pertinent field, by the use of a suitable restriction enzyme site.

20 Particularly, the recombinant vector of the present invention is an expression vector wherein a DNA encoding SLDH is disposed under the control of a promoter functional in a certain host cell. Usable vector is not particularly restricted as long as it contains a promoter region
25 functional in various host cells such as procaryotic and/or eucaryotic cells and is capable of controlling the

20

initiation codon, ATG is generally used. In some cases, GTG can be also used. As the stop codon, conventional TGA, TAA and TAG can be used.

When a DNA encoding the SLDH of the present invention
5 is prepared from a genomic DNA derived from a cell or tissue
that produces the enzyme, and obtained in a form containing
inherent promoter and terminator regions, and the expression
vector of the present invention can be prepared by inserting
the DNA into a suitable site of a known cloning vector that
10 can replicate/maintain or autonomously proliferate in a host
cell to be transformed. The usable cloning vector in the
case where the host is bacteria is exemplified by pBR vector,
pUC vector and the like derived from *Escherichia coli*,
pUB110, pTP5 and pC194, derived from *Bacillus subtilis*, and
15 the like.

When an expression vector containing a DNA encoding the SLDH of the present invention is used for the production of a recombinant SLDH, particularly when the SLDH is extremely unstable and typical purification method may cause
20 deactivation of the enzyme on the way of purification, the use of an expression vector containing a modified SLDH coding sequence, as in the following, is particularly preferable. The modified SLDH coding sequence comprises, a sequence wherein a base sequence encoding a specific amino acid
25 sequence capable of accelerating the purification of SLDH is added to the terminus of the SLDH coding sequence to allow

expression of SLDH in which the specific amino acid sequence has been added to the terminus of the original SLDH amino acid sequence. The specific amino acid sequence capable of accelerating the purification of SLDH is exemplified by amino acid sequence capable of adsorbing to a metal ion chelate, preferably a sequence consisting of basic amino acids such as histidine, lysine, arginine and the like, more preferably a sequence consisting of histidine. Such sequence can be added to the terminus of amino or carboxyl of SLDH, with preference given to addition to the carboxyl terminus. Such modified SLDH coding sequence can be constructed by synthesizing an oligonucleotide wherein a base sequence encoding the amino acid sequence to be added is added to a base sequence consistent with a terminus sequence of the inherent SLDH coding sequence, and, using this as one of the primers and SLDH DNA as a template, performing PCR. The resulting recombinant SLDH can be quickly isolated and purified using a carrier on which a metal ion chelate capable of adsorbing the added amino acid sequence has been immobilized, as detailed in the following.

When an expression vector containing a DNA encoding the SLDH of the present invention is used for the production of 2KLGA, an expression vector containing, in addition to the DNA, a DNA encoding SDH and/or SNDH in a form permitting expression in the host cell may be used. The DNA encoding SLDH, a DNA encoding SDH and a DNA encoding SNDH may be

placed under control of different promoters, or two of which or more may be placed in tandem under the control of the same promoter.

The transformant of the present invention can be
 5 prepared by transforming a host cell with a recombinant vector containing a DNA encoding the SLDH of the present invention. The host cell is not particularly limited as long as it can be adapted to the recombinant vector to be used and can be transformed, and various cells conventionally used in
 10 this field, such as a naturally occurring cell or an artificially produced mutant cell or a recombinant cell, can be utilized. Preferably, bacteria, particularly *Escherichia coli* (e.g., DH5 , HB101 and the like), *Bacillus subtilis*, the genus *Pseudomonas* bacteria (e.g., *Pseudomonas fluorescence*
 15 and the like), the genus *Gluconobacter* bacteria (e.g., *Gluconobacter oxydans* and the like), the genus *Pseudogluconobacter* bacteria, the genus *Acetobacter* bacteria and the like are used.

A recombinant vector can be introduced into a host cell
 20 by a method conventionally known. For example, when the host is bacteria such as *Escherichia coli*, *Bacillus subtilis* and the like, the method of Cohen et al. [*Proc. Natl. Acad. Sci. USA*, 69: 2110 (1972)], protoplast method [*Mol. Gen. Genet.*, 168: 111 (1979)], competent method [*J. Mol. Biol.*, 56: 209
 25 (1971)], electroporation method and the like can be used.

Particularly, the transformant of the present invention

is a host cell transformed with an expression vector containing a DNA encoding the SLDH of the present invention. When the transformant is prepared with the aim of producing 2KLGA from D-sorbitol, the host cell needs to have an ability to convert L-sorbose to 2KLGA. Preferably, the host cell produces SDH and SNDH activity. Such naturally occurring cell is, for example, bacteria belonging to the genus *Gluconobacter*, the genus *Acetobacter*, the genus *Pseudogluconobacter* and the like, specifically *Gluconobacter oxydans* T-100 (FERM BP-4415; International Patent Publication No. WO95/23220) and the like. Such artificially prepared cell is, for example, a cell transformed with an expression vector functionally containing a DNA encoding SDH and SNDH isolated from the above-mentioned naturally occurring bacteria and the like, preferably *Escherichia coli*, the genus *Pseudomonas* bacteria, the genus *Gluconobacter* bacteria, the genus *Pseudogluconobacter* bacteria, the genus *Acetobacter* bacteria and the like. Specifically, *E. coli* JM109-pUC19SD5 (International Patent Publication No. WO94/20609), *Gluconobacter oxydans* NB6939-pSDH-tufB1, *Gluconobacter oxydans* NB6939-pSDH-trp6, *Gluconobacter oxydans* NB6939-pSDH-PL1, *Gluconobacter oxydans* NB6939-pSDH-tac8 (all from International Patent Publication No. WO95/23220) and the like are exemplified.

The transformant of the present invention can be also obtained by transforming a host cell with an expression

vector containing, in addition to the above-mentioned DNA encoding SLDH, a DNA encoding SDH and/or a DNA encoding SNDH in a form permitting expression in the host cell. When the expression vector lacks one of the DNA encoding SDH and the DNA encoding SNDH, the host cell may be co-transformed along with a different expression vector containing said DNA.

The recombinant SLDH of the present invention can be produced by culturing a transformant containing an expression vector containing a DNA encoding the above-mentioned SLDH in a suitable medium and harvesting SLDH from the obtained culture.

The nutrient medium to be used contains, as a carbon source, saccharides such as glucose and fructose, glycerol, preferably L-sorbose and D-sorbitol. It may contain an inorganic or organic nitrogen source (e.g., ammonium sulfate, ammonium chloride, hydrolysate of casein, yeast extract, polypeptone, bactotrypton, beef extract and the like). When desired, other nutrient sources [e.g., inorganic salt (e.g., sodium diphosphate or potassium diphosphate, potassium hydrogenphosphate, magnesium chloride, magnesium sulfate, calcium chloride), vitamins (e.g., vitamin B1), antibiotics (e.g., ampicillin, kanamycin) etc.] may be added to the medium. Preferably, the medium contains D-sorbitol, yeast extract, CaCO_3 and glycerol as ingredients. The medium has a sugar (D-sorbitol) concentration of generally 1 - 50%, preferably 2 - 40%.

A transformant is cultured at generally pH 5.5 - 8.5, preferably pH 6 - 8, at generally 18 - 40°C, preferably 20 - 35°C for 5 - 150 h.

SLDH can be purified by appropriately combining various separation techniques typically used according to the fraction having an SLDH activity. Since the SLDH of the present invention is NAD(P)⁺ dependent, it highly likely localizes in a soluble fraction of the transformant. In this case, after the completion of the culture, the culture is
5
10 filtrated or centrifuged to recover the cell, which is then ruptured by ultrasonication, lysozyme treatment, osmotic pressure shock and the like to give a cell extract for use.

When the recombinant SLDH is produced in the aforementioned form wherein a specific amino acid sequence is
15 added to the terminus, the SLDH can be quickly and easily purified by a treatment including chromatography using a carrier, on which a metal ion chelate capable of adsorbing the specific amino acid sequence is immobilized (immobilized metal affinity chromatography; IMAC). The metal ion chelate
20 adsorber to be used can be prepared by bringing a solution containing a transition metal (e.g., divalent ion such as cobalt, copper, nickel and iron, trivalent ion such as iron, aluminum and the like, preferably divalent ion of cobalt) into contact with a matrix to which a ligand, for example,
25 iminodiacetate group, nitrilotriacetate group, tris(carboxymethyl)ethylenediamine group and the like, has

According to a different production method of 2KLGA of the present invention, a host cell capable of converting L-sorbose to 2KLGA, which is transformed with an expression vector containing a DNA encoding the above-mentioned SLDH, is
 5 cultured in a suitable medium, and D-sorbitol is brought into contact with the obtained culture, or, when the SLDH, SDH and SLDH activity is present in an intracellular fraction of the host cell, with a cell extract thereof, to give 2KLGA. The method for bringing D-sorbitol into contact with the culture
 10 includes culture of the host cell in a medium containing D-sorbitol.

The medium and culture conditions to be used for the production method of L-sorbose and the production method of 2KLGA of the present invention may be the same as or
 15 partially different from those used for the above-mentioned production method of SLDH.

When D-sorbitol or L-sorbose is brought into contact with a cell extract, the culture after the completion of culture is centrifuged or filtrated to recover the cell,
 20 which is suspended in a suitable buffer, such as acetate buffer, and the cell is ruptured by ultrasonication and the like and subjected to a centrifugation treatment to give a supernatant which can be used as a cell extract.

The L-sorbose or 2KLGA thus produced can be purified
 25 from a reaction mixture (when the transformant is cultured in a medium containing D-sorbitol or L-sorbose, a culture

precipitation. This was subjected to dephosphorylation with 3 U calf intestine-derived alkaline phosphatase (CIAP) at 37°C for 1 hour and purified by ethanol precipitation.

Separately, the chromosomal DNA (100 µg) of the strain *G.*

5 *oxydans* G624 obtained in the above-mentioned (1) was

partially digested with 5 U Sau3AI at 37°C for 1 minute, and purified by ethanol precipitation. The partial digest (ca.

1.5 µg) and BamHI digest of pcos6EMBL (ca. 3 µg) were ligated with 3 U T4 DNA ligase at 4°C for 16 hours. A portion (3 µl)

10 thereof was subjected to *in vitro* packaging using GIGAPACK II Gold Packaging Extract (STRATAGENE). This packaging solution

was diluted 50-fold with SM buffer [50 mM Tris-HCl (pH 7.5) - 100 mM NaCl - 8 mM MgSO₄ - 0.1% gelatin] and 25 µl of the indicating bacteria (*Escherichia coli* XL1-Blue MRA) was

15 infected with 25 µl thereof, sown on a 50 µg/ml kanamycin-containing LB plate and stood at 37°C overnight. About 400 colonies were obtained, which means a cosmid library of about 400000 clones was obtained.

(3) Screening of clone having SLDH activity

20 In a 96 well plate rounded bottom (Nalge) containing a

0.9-fold diluted LB medium containing 5% sorbitol and 50

µg/ml kanamycin by 150 µl per well, 368 cosmid clones were

cultured with gentle shaking at 30°C for 3 days. After

centrifugation (2,000 rpm, 10 minutes), 0.5 mg/ml resorcin-

25 ethanol solution (30 µl) and 0.216 mg/ml ferric sulfate (III)

ammonium-hydrochloric acid solution (30 μ l) were added to the culture supernatant (20 μ l), and the mixture was heated at 80°C for 1 hour. Using a medium alone similarly reacted as a control, 3 clones of 1A4, 1A5, 4A9 which showed deeper brown color than did the control were selected as the clones having a conversion capability to sorbose (fructose). The culture supernatants thereof were analyzed by HPLC [column: Polyspher OA KC (E. Merck), 7.8×300 mm; temperature: room temperature; migration phase: 0.01N H₂ SO₄ ; flow amount: 0.4 ml/minute; detection: RI] and sorbose was detected for each clone. Thus, these 3 clones were considered to have an SLDH activity. The length of the insert part of these cosmid clones was about 40 kb for all of them.

(4) Subcloning to charomid vector (downsizing of insert)

The cosmid clone 1A4 (300 ng) having an SLDH activity was partially digested with 20 mU Sau3AI at 37°C for 1 hour. The charomid 9-28 (1 μ g, Nippon Gene) was digested with 4 U BamHI at 37°C for 1 hour. These two solutions were mixed, purified by ethanol precipitation, dissolved in 2-fold diluted TE buffer (5 μ l) and ligated with 1 U T4 DNA ligase at 4°C for 16 hours. One(1) μ l thereof was subjected to *in vitro* packaging using GIGAPACK II XL Packaging Extract (STRATAGENE). The packaging solution (75 μ l) and SM buffer (75 μ l) were mixed and used for infecting 150 μ l of indicating bacteria (*Escherichia coli* DH-1), which was sown

on a 50 µg/ml ampicillin-containing LB plate and incubated at 37°C for 1 day. Of the colonies appeared, 95 colonies were cultured with gentle shaking at 30°C for 3 days in a 96 well plate rounded bottom (Nalge) containing a 0.9-fold diluted LB medium containing 5% sorbitol and 50 µg/ml kanamycin by 150 µl per well. After centrifugation (2,000 rpm, 10 minutes), 0.5 mg/ml resorcin-ethanol solution (30 µl) and 0.216 mg/ml ferric sulfate (III) ammonium-hydrochloric acid solution (30 µl) were added to the culture supernatant (20 µl), and the mixture was heated at 80°C for 1 hour. Using a medium alone similarly reacted as a control, 6 clones of G1, C2, A4, B7, H10, B12 which showed deeper brown color than did the control were selected as the clones having a conversion capability to sorbose. The length of the insert part of these charomid clones was about 15 kb for all of them.

(5) Subcloning of SLDH gene to plasmid vector

From the restriction enzyme map of the clones obtained so far, it was found that SLDH gene did not have a SacI site or a XbaI site. Thus, 1 µg of charomid B7 was digested with 10U of SacI and 10U of XbaI to give about 6 kb (B7SX3) and about 9 kb (B7SX2) SacI-XbaI fragments. These two fragments were respectively ligated with *Escherichia coli*-*Pseudomonas* shuttle vector pUCP19 [1.8 kb PstI fragment derived from pRO1614 was inserted into NarI site of pUC19 and purified from *Escherichia coli* DH5 αF' (ATCC 87110)] and transformed

with *Pseudomonas* (this strain was later named *Pseudomonas* sp. F-1, hereinafter to be referred to by this designation) by the electroporation method to give Ps./pUCP19-B7SX3 and Ps./pUCP19-B7SX2. The preparation of competent cell and
5 conditions of transformation followed those of *Escherichia coli*. These two clones were cultured in a medium containing sorbitol. As a result, sorbose conversion capability was found in Ps./pUCP19-B7SX2. Therefore, Ps./pUCP19-B7SX2 was cultured in a medium (pH 7.4) containing 5% sorbitol, 1%
10 bactotrypton, 0.5% yeast extract, 1% sodium chloride and 50 µg/ml ampicillin at 30°C for 4 days to give 2.4 mg/ml of sorbose (conversion efficiency: 5%). This sorbose was separated by HPLC and the coincidence of retention time with the standard product was confirmed. HPLC was performed under
15 the same conditions as in the above-mentioned (3). Using GC/MS [column:DB-5 (J & W Scientific), 0.32 mm × 30 m (film 0.25 µm); temperature: injection=230°C, column=100°C (5 minutes) → heating at 10°C/minute for 10 minute → 200°C (5 minutes) → heating at 30°C/minute for 1 minute → 230°C (4
20 minutes), detect=230°C; flow amount: pressure control 20 kPa(He)], the coincidence of mass pattern with the standard product was confirmed.

(6) Determination of base sequence of SLDH gene

The restriction enzyme map of the insert part of
25 plasmid pUCP19-B7SX2 of transformant of *Pseudomonas*, Ps./pUCP19-B7SX2, that expresses an SLDH activity was assumed

as shown in FIG. 2. By digestion of 1 µg of pUCP19-B7SX2 with 10U of Hind III at 37°C for 1 hour, about 4 kb Hind III-Hind III fragment was obtained. This Hind III-Hind III fragment was ligated with vector pUCP19 and plasmid pUCP19-HC was constructed. *Pseudomonas* was transformed with this plasmid to give Ps./pUCP19-HC. This transformant was cultured in a medium containing sorbitol. As a result, expression of SLDH activity was acknowledged. Thus, this Hind III-Hind III fragment was found to contain full length SLDH gene. The base sequence of this about 4 kb Hind III-Hind III fragment was determined. First, the insert part of pUCP19-HC was divided into about 1.1 kb SphI-SphI fragment (S1), about 0.8 kb EcoRI-SphI fragment (ES) and about 1.3 kb EcoRI-EcoRI (E1) fragment (FIG. 2), and each was subcloned to pUC18 to give pUC18-S1, pUC18-ES and pUC18-E1.

Using plasmids pUCP19-HC, pUC18-S1, pUC18-ES and pUC18-E1 as templates and using universal primer and reverse primer (New England Labs.), which were M13 sequencing primers, first sequencing was performed. The sample was fluorescent labeled with BigDye Terminator Cycle Sequencing kit (Applied Biosystems) and analyzed with ABI PRISM 310 Genetic Analyzer (Applied Biosystems). The following 11 kinds of primers were synthesized and using pUCP19-HC as a template sequencing was performed, whereby the base sequence of about 4 kb Hind III-Hind III fragment was determined (Sequence Listing SEQ ID NO:2).

SLDH gene sequencing primer

SL1 GCTGCTGAGTGATCCG (Sequence Listing SEQ ID NO:3)

SL2 GACTGCTACTTCGATCC (Sequence Listing SEQ ID NO:4)

SL3 CCTACACCTAGCCTGC (Sequence Listing SEQ ID NO:5)

5 SL4 CAGTGCCGTCATGAGG (Sequence Listing SEQ ID NO:6)

SL5 TCCTGATCTCGGTGCG (Sequence Listing SEQ ID NO:7)

SL6 GATGCTTCAGCACGGC (Sequence Listing SEQ ID NO:8)

SL7 GACGATCACGGAAGGC (Sequence Listing SEQ ID NO:9)

SL8 GGTTACGTGGTCGAGG (Sequence Listing SEQ ID NO:10)

10 SL9 CTATACGTGACAGGTCC (Sequence Listing SEQ ID NO:11)

SL10 GCGCGATCTGGATACG (Sequence Listing SEQ ID NO:12)

SL11 CGAGGATCTCGAACGG (Sequence Listing SEQ ID NO:13)

From the analysis of the base sequence, 1455 bp ORF was found (base number 537 - 1991). Therefore, SLDH was assumed to consist of 485 amino acids and has a molecular weight of about 54 kDa. As a result of the homology search, it showed 42% homology with mannitol dehydrogenase of *Pseudomonas fluorescence*.

20 **Example 2** Production of recombinant SLDH

(1) construction of plasmid expressing SLDH having histidine-tag (hereinafter to be referred to as His-tagged SLDH)

For purification of the recombinant protein, a tag system utilizing 6xhistidine was an extremely easy method. That is, a protein having 6 histidine tag is expressed, and utilizing interaction of a metal (e.g., cobalt, nickel) and

and SacI site (underlined)]

TAGCTGAGCTCATGGGACAGATCTGAGC (Sequence Listing SEQ ID
NO:17)

The about 360 bp fragment specifically amplified in PCR
1 was digested with NheI and BamHI, and the about 100 bp
fragment specifically amplified in PCR 2 was digested with
BamHI and SacI. Separately, the about 2 kb fragment obtained
by digesting pUCP19-HC with BglII and PstI was inserted into
BamHI-PstI fragment of pUCP19 to give plasmid pUCP19-SLDH
10 (FIG. 3) wherein the downstream of BglII site of the insert
was removed. This was digested with NheI and SacI, and the
obtained about 6.2 kb fragment was ligated with the above-
mentioned two PCR amplification fragments with T4 DNA ligase
to construct pUCP19-SLDH-His. *Pseudomonas* was transformed
15 with this plasmid to give Ps./pUCP19-SLDH-His.

(2) Purification of His-tagged SLDH

One loopful of cryopreservation stock of transformant
Ps./pUCP19-SLDH-His was inoculated to LB medium (2 ml)
containing 50 µg/ml ampicillin in a 15 ml centrifuge tube
20 (Corning) and cultured at 30°C for 16 hours. The 1.5 ml
thereof was inoculated to LB medium (50 ml) containing 5%
sorbitol and 50 µg/ml ampicillin in a 500 ml Erlenmeyer flask
and cultured at 25°C for 3 days. The cells were harvested by
centrifugation (6,000 rpm, 4°C for 5 minutes), and suspended
25 in 10 ml of 100 mM NaCl-containing 20 mM Tris-HCl (pH 8.0).
The suspension was treated with an ultrasonication

homogenizer (Tomy UD-201) for 5 minutes (50% interval),
centrifuged (15,000 rpm, 4°C for 10 minutes) and a
supernatant was recovered to give a cell-free extract. TARON
resin (2 ml, CLONTECH) was placed in a 15 ml centrifuge tube
5 (Corning), and washed twice with 10 ml of 100 mM NaCl-
containing 20 mM Tris-HCl (pH 8.0) for equilibration. The
above-mentioned cell-free extract (5 ml) was added and the
mixture was shaken at room temperature for 20 minutes to
adsorb His-tagged SLDH, followed by washing 3 times with 100
10 mM NaCl-containing 20 mM Tris-HCl (10 ml, pH 8.0) over 10
minutes. 100 mM NaCl-containing 20 mM Tris-HCl buffers (2
ml, pH 8.0) respectively containing 10 mM, 30 mM, 50 mM and
100 mM imidazole were added successively, and shaken at room
temperature for 2 minutes to elute His-tagged SLDH. As a
15 result, SLDH activity eluted in a 30 mM - 50 mM imidazole
fraction. This fraction was applied to SDS-PAGE analyze to
detect nearly single band.

(3) Analysis of N terminus amino acid sequence

The His-tagged SLDH purified in the above-mentioned (2)
20 was electrophoresed using a multigel (Daiichi Pure Chemicals)
having a gel concentration of 12.5% with 40 mA current over 1
hour, and using Horiz-Blot (Atto), transferred onto a PVDF
membrane (Immobilon PSQ; Millipore). The membrane was
stained with coomassie brilliant blue G-250, and a band
25 seemingly an about 55 kDa SLDH was cut out with a pair of
scissors. This PVDF membrane was subjected to amino acid

(2.1 μ g protein) prepared in Example 2(4) and distilled water (0.29 ml) was added to 500 mM sorbitol (0.4 ml) to start the reaction (25°C), and increase in NADH (or NADPH) was measured by a spectrophotometer (UV-2200; Shimadzu) based on absorbance at 340 nm as an index. For the reaction solutions having pH 10.1 and pH 9.0, glycine/NaOH buffer was used, and for reaction solutions having pH 8.0 and pH 7.0, potassium phosphate buffer was used. The enzyme activity (1 unit) was defined to be an amount to generate 1 μ mol of NADH (or NADPH) per minute. The molecular extinction coefficient of NAD(P)H was 6.3 $\text{mM}^{-1} \text{cm}^{-1}$. The protein amount was measured with bovine serum albumin (BSA) as a standard by the Lowry method. As a result, SLDH could utilize both NAD^+ and NADP^+ as coenzymes, and NADP^+ showed higher specificity. The activity of this enzyme was higher in the alkaline pH (Table 1).

Table 1

Coenzyme	pH	Activity (U/mg protein)
NADP^+	10.1	130.2
	9.0	30.0
	8.0	22.9
	7.0	4.2

NAD ⁺	10.1	8.1
	9.0	3.4
	8.0	1.2
	7.0	0.1

(2) Substrate specificity

In the same manner as in the above-mentioned (1) except that the reaction solution contained various substrates to replace sorbitol, the buffer was glycine/NaOH buffer (pH 10.1), and coenzyme was NADP⁺, the SLDH activity was measured. As a result, this enzyme could utilize, besides sorbitol, mannitol and arabitol as a substrate, but showed no action on xylitol, ribitol, inositol or glycerol (Table 2).

10

Table 2

Substrate	Activity (U/mg protein)
sorbitol	130.2
mannitol	85.7
arabitol	88.1
xylitol	0
ribitol	0
inositol	0
glycerol	0

(3) Michaelis constant

Using sorbitol as a substrate, SLDH activity was measured according to the method of the above-mentioned (2). As a result, the K_m value for sorbitol was 132 mM (25°C).

5 **Example 4** Preparation of *Pseudomonas* transformant having SNDH/SDH expression vector and study of 2KLGA productivity by this transformant

Of the pBBR plasmids that are broad host range plasmids [Gene, 166, 175 (1995)] supplied by Dr. Kovach at Louisiana State University, Medical Center, SNDH/SDH gene was introduced into the genus *Pseudomonas* strain using pBBR1MCS-2 (kanamycin resistant) and pBBR1MCS-3 (tetracycline resistant) as vectors, and fermentative production of 2KLGA from L-sorbose by the obtained transformant was studied.

15 (1) Construction of SNDH/SDH expressing broad host range plasmid

Plasmid pSDH-tufB1-Eco-d9U (FIG. 4) (5 µg) containing SNDH/SDH gene and using tufB as a promoter was digested with EcoRI (50U, Behringer-Mannheim) at 37°C for 1 hour, and electrophoresed on 0.8% agarose gel, which was followed by separation of a 3.7 kb EcoRI/EcoRI fragment containing SNDH/SDH gene. This fragment was inserted into the EcoRI site of pBBR1MCS-2. The plasmid inserted in the same direction as the β-galactosidase gene was taken as pBBR (Km)-SDH SNDH (FIG. 5).

Plasmid pSDH-tufB1 (10 µg, construction method is

(3) Transformation of *Pseudomonas*

The competent cells of *Pseudomonas* cryopreserved in liquid nitrogen sp.F-1 were thawed in ice water, and solutions of an SNDH/SDH expressing broad host range plasmid constructed in the above-mentioned (1), pBBR(Km)-SDH· SNDH and pBBR(Tc)-SDH· SNDH, were added by 1 μ l (ca. 1 μ g) each, and stood at 4°C for 30 minutes. This was transformed using a Gene Pulser gene transfer device (Bio-Rad) in a cuvette having a distance of 0.1 cm between electrodes under the conditions of 200 Ω , 1.8 kV, 25 μ F, and suspended in L medium containing 0.4% glucose, which was followed by shaking at 30°C for 1 hour. They were sown on an L agar plate containing 50 μ g/ml kanamycin and an L agar plate containing 20 μ g/ml tetracycline, cultured at 30°C for 2 days to give transformant Ps./pBBR(Km)-SDH· SNDH and Ps./pBBR(Tc)-SDH· SNDH.

(4) Fermentative production of 2KLGA from sorbose by the transformant

A single colony of transformants Ps./pBBR(Km)-SDH· SNDH and Ps./pBBR (Tc)-SDH· SNDH obtained in the above-mentioned (3) was each inoculated to 5 ml of L medium in a 16.5×165 mm test tube and cultured at 30°C for 2 days. The culture solution (0.5 ml) was inoculated to a medium (10 ml, pH 7.4) for 2KLGA production containing 5% sorbose, 0.1% glucose, 0.9% bactotrypton (Difco), 0.45% yeast extract (Difco), 0.9%

sodium chloride and 2% calcium carbonate in a 100 ml
 Erlenmeyer flask, and cultured at 30°C for 5 days. The
 culture solution was separated by centrifugation and sorbose,
 sorbosone, 2KLGA and L-idonic acid in the culture supernatant
 5 were quantitatively determined. The sorbose, sorbosone,
 2KLGA and L-idonic acid were each quantitatively determined
 by HPLC under the following conditions.

[sorbose]

column: Polyspher OA KC (7.8 mm inner diameter×300 mm;

10 Cica-MERCK)

migration phase: 0.01N H₂SO₄

column temperature: room temperature

flow rate: 0.4 ml/minute

detection: differential refractometer

15 [sorbosone (post-column labeling method)]

column: Polyspher OA KC (7.8 mm inner diameter×300 mm; Cica-
 MERCK)

migration phase (labeling agent): 0.04M benzamidine

hydrochloride

20 0.25M potassium sulfite

2 mM boric acid/0.1N potassium hydroxide

flow rate: 0.3 ml/minute

detection: fluorescent detector (excitation wavelength: 315
 nm, detection wavelength: 405 nm)

25 [2KLGA and L-idonic acid]

column: Capcell pak NH2 (4.6 mm inner diameter×250 mm;

For comparison, production of 2KLGA and L-idonic acid by a non-transformant *Pseudomonas* sp. F-1 was also investigated. The glycerol cryopreserved cells of *Pseudomonas* sp. F-1 were inoculated to 5 ml of L medium in a 16.5x165 mm test tube and cultured at 30°C for 1 day. The culture solution (1 ml) was inoculated to a medium (10 ml, pH 7.4) containing 5% sorbose, 0.9% bactotrypton (Difco), 0.45% yeast extract (Difco) and 0.9% sodium chloride in a 100 ml Erlenmeyer flask, and cultured at 30°C for 3 days. The culture solution was centrifuged and sorbose, sorbosone, 2KLGA and L-idonic acid in the culture supernatant were similarly determined quantitatively. As a result, sorbose was consumed (5.7 mg/ml) but sorbosone, 2KLGA and L-idonic acid were not detected.

From the above, by the introduction of SNDH/SDH gene into 2KLGA and L-idonic acid non-producing *Pseudomonas* sp. F-1, a transformant that highly produces 2KLGA and L-idonic acid from sorbose could be obtained.

Example 5 Preparation of *Pseudomonas* transformant containing SNDH/SDH expression vector and consideration of 2KLGA productivity of the transformant - (2)

In the same manner as in Example 4 and using a different strain [strain *Pseudomonas* IF03309; supplied by the Institute for Fermentation, Osaka (17-85, Juso-honmachi 2-chome, Yodogawa-ku, Osaka)] belonging to the genus

Pseudomonas as a host, a transformant was prepared, into which the SNDH/SDH gene was introduced, and the 2KLGA and L-idonic acid productivity of this transformant was investigated.

5 (1) Introduction of SNDH/SDH gene into strain *Pseudomonas* IFO3309

Glycerol cryopreserved cells of the strain *Pseudomonas* IFO3309 were treated in the same manner as in Example 4(2) to prepare cryopreserved cells of competent cells. The
10 competent cells cryopreserved in liquid nitrogen were thawed in ice water, and a solution 1 μ l (ca. 1 μ g) of Ps./pBBR(Km)-SDH⁺ SNDH, which was an SNDH/SDH expressing broad host range plasmid, was added and the mixture was stood at 4°C for 30 minutes. This was transformed using a Gene Pulser gene
15 transfer device (Bio-Rad) under the same conditions as in Example 4(3) to give transformant Ps. IFO3309/pBBR(Km)-SDH⁺ SNDH.

(2) Fermentative production of 2KLGA by the transformant

One loopful of the Ps. IFO3309/pBBR(Km)-SDH⁺ SNDH
20 obtained in the above (1) was inoculated to a medium (5 ml, pH 7.0) containing 2% sorbitol and 0.5% yeast extract (Difco) in a 16.5x165 mm test tube, and cultured at 28°C for 1 day. The culture solution (1 ml) was inoculated to a medium (10 ml, pH 7.0) containing 5% sorbitol, 0.5% yeast extract
25 (Difco), 0.2% polypeptone (Wako Pure Chemical Industries), 0.1% K₂ HPO₄, 0.5% MgSO₄ · 7H₂O and 2%CaCO₃ in a 100 ml

Erlenmeyer flask and cultured at 28°C for 7 days. The culture solution was centrifuged and in the same manner as in Example 4(4), sorbitol, sorbose, sorbosone, 2KLGA and L-idonic acid in the culture supernatant were quantitatively
 5 determined. For comparison, non-transformant the strain *Pseudomonas* IFO3309 was cultured under the same conditions and sorbitol, sorbose, sorbosone, 2KLGA and L-idonic acid in the culture supernatant were quantitatively determined.

As a result, by the non-transformant, sorbitol was
 10 consumed (0.4 mg/ml), sorbose was produced (3.9 mg/ml) but sorbosone, 2KLGA and L-idonic acid were not detected. On the other hand, 2KLGA (1.2 mg/ml) and L-idonic acid (0.5 mg/ml) were produced by the transformant Ps. IFO3309/pBBR(Km) - SDH: SNDH (Table 4). In other words, it was confirmed that,
 15 by the introduction of SNDH/SDH gene into this host, the ability to produce 2KLGA and L-idonic acid from sorbitol was imparted even under the conditions where *Pseudomonas* IFO3309 cannot produce 2KLGA or L-idonic acid.

carbonate at 30°C for 4 days to give 1.1 mg/ml of 2KLGA and 1.7 mg/ml of idonic acid. The 2KLGA was separated by HPLC and coincidence with a standard product in the retention time was confirmed. Using GC/MS, coincidence with a standard product in the mass pattern was confirmed. HPLC and GC/MS were performed under the same conditions as in Example 1(3) and (5).

Example 7 Preparation of various *Pseudomonas* transformants

(1) Ps./pUCP19-SLDH+pBBR(Km)-SDH• SNDH

Pseudomonas sp.F-1 was transformed with pUCP19-SLDH constructed in Example 2(1) to give Ps./pUCP19-SLDH. pBBR(Km)-SDH• SNDH was further introduced into this recombinant *Pseudomonas* to give Ps./pUCP19-SLDH+pBBR(Km)-SDH• SNDH.

(2) Ps./pUCP19-SLDH-tufB+pBBR(Km)-SDH• SNDH

To introduce SspI site into the upstream of initiation codon of SLDH gene, PCR was performed using pUCP19-SLDH (5 µg) as a template in the presence of the following primers (20 pmol each) using pfu DNA polymerase (2.5 U) (94°C, 30 seconds→55°C, 2 minutes→72°C, 2 minutes, 25 cycles).

sense primer [including SspI site (underlined) and sequence identical with 5' terminus of SLDH coding sequence]

TAGGAATATTTCTCATGATTACGCGCGAAACCC (Sequence Listing SEQ

ID NO:19)

antisense primer [sequence identical with sequence

downstream of EagI site in SLDH coding sequence]

GATGCTTCAGCACGGC (Sequence Listing SEQ ID NO:20)

The about 360 bp fragment specifically amplified by PCR was digested with SspI and EagI. pUCP19-SLDH was digested with PstI and EagI to give about 5.7 kb fragment. These two fragments and PstI-SspI fragment (Sequence Listing SEQ ID NO:21) containing tufB promoter were ligated with T4 DNA ligase to construct pUCP19-SLDH-tufB. *Pseudomonas* was transformed with this plasmid to give Ps./pUCP19-SLDH-tufB. Furthermore, pBBR(Km)-SDH⁺ SNDH capable of expressing SNDH/SDH activity was introduced to give Ps./pUCP19-SLDH-tufB+pBBR(Km)-SDH⁺ SNDH.

(3) Ps./pUCP19-3DH

pUCP19-SLDH-tufB (5 µg) was digested with 40 U KpnI and 40 U PstI at 37°C for 1 hour to give a 1.6 kb fragment. pUCP19-SDH⁺ SNDH (expression vector obtained by incorporating SNDH/SDH gene derived from *G. oxydans* T-100 into pUCP19; FIG. 7) (1 µg) was digested with 10 U KpnI and 10 U PstI at 37°C for 1 hour to give a 8.2 kb fragment. These two fragments were ligated with T4 DNA ligase to construct pUCP19-3DH. *Pseudomonas* was transformed with this plasmid to give Ps./pUCP19-3DH.

Example 8 Consideration of productivity of 2KLGA

Since production of 2KLGA by recombinant *Pseudomonas* was confirmed, the productivity of 2KLGA was studied in media

culture		sorbitol	sorbose	sorbose	2KLGA	idonic acid
1		44.1	6.3	0.1	3.7	1.6
2		44.8	3.1	0	4.8	2.2
3		26.7	5.1	0	10.9	8.4
4		26.6	0	0	9.0	ND
5		26.6	0	0	10.7	ND
6		30.7	5.2	0	7.5	ND
7	days	sorbitol	sorbose	sorbose	2KLGA	idonic acid
	1	41.1	0	0	4.2	ND
	3	25.6	0	0	10.6	ND
	5	14.2	0	0	16.3	ND
	7	7.6	0	0	18.4	15.5

(unit: mg/ml)

ND: Not determined

5 **Example 9** Fermentative production of sorbose or 2KLGA by *Pseudomonas putida* transformant into which SLDH expression vector and/or SNDH/SDH expression vector were introduced

In the following test, the preparation and transformation of competent cells of the strain *Pseudomonas*
 10 *putida* IF03738 followed the above-mentioned *Pseudomonas* sp.F-1. Because the strain *Pseudomonas putida* IF03738 is ampicillin resistant, when ampicillin resistance is used as a

selection marker, cells were sown on an L agar plate containing 500 µg/ml ampicillin (10-fold amount of normal level) after electroporation, and cultured at 30°C for 1 day to pick up large colonies for the selection of transformant.

- 5 (1) Fermentative production of sorbose from sorbitol by transformant into which SLDH expression vector was introduced

SLDH gene (pUCP19-SLDH) was introduced into the strain *Pseudomonas putida* IF03738. A single colony of the obtained transformant *Pseudomonas putida* IF03738/pUCP19-SLDH was
 10 inoculated to a medium (10 ml, pH 7.4) for sorbose production, which contained 5% sorbitol, 0.9% bactotrypton (Difco), 0.45% yeast extract (Difco), 0.9% sodium chloride and 500 µg/ml ampicillin in a 100 ml Erlenmeyer flask and cultured at 30°C for 3 days. The culture solution was
 15 centrifuged and sorbitol and sorbose in the culture supernatant were quantitatively determined. As a result, 34.6 mg/ml of sorbitol remained and 7.6 mg/ml of sorbose was generated.

- (2) Fermentative production of 2KLGA from sorbose by
 20 transformant into which SNDH/SDH expression vector was introduced

SNDH/SDH gene (pBBR(Km)-SDH·SNDH) was introduced into *Pseudomonas putida* IF03738. A single colony of the obtained transformant *Pseudomonas putida* IF03738/pBBR(Km)-SDH·SNDH was
 25 inoculated to a medium (10 ml, pH 7.4) for 2KLGA production, which contained 5% sorbose, 0.9% bactotrypton (Difco), 0.45%

yeast extract (Difco), 0.9% sodium chloride, 2% calcium carbonate and 50 µg/ml kanamycin in a 100 ml Erlenmeyer flask and cultured at 30°C for 7 days. The culture solution was centrifuged and sorbose, 2KLGA and idonic acid in the culture supernatant were quantitatively determined. As a result, 34.3 mg/ml of sorbose remained and 13.9 mg/ml of 2KLGA and 3.5 mg/ml of idonic acid were generated.

(3) Fermentative production of 2KLGA from sorbitol by transformant into which SLDH expression vector and SNDH/SDH expression vector were introduced

SLDH and SNDH/SDH genes (pUCP19-SLDH and pBBR(Km)-SDH~~H~~ SNDH) were introduced into the strain *Pseudomonas putida* IFO3738. A single colony of the obtained transformant *Pseudomonas putida* IFO3738/pUCP19-SLDH+pBBR(Km)-SDH~~H~~ SNDH was inoculated to a medium (10 ml, pH 7.4) for 2KLGA production, which contained 5% sorbitol, 0.9% bactotrypton (Difco), 0.45% yeast extract (Difco), 0.9% sodium chloride, 2% calcium carbonate, 500 µg/ml ampicillin and 50 µg/ml kanamycin in a 100 ml Erlenmeyer flask and cultured at 30°C for 7 days. The culture solution was centrifuged and sorbitol, sorbose, 2KLGA and idonic acid in the culture supernatant were quantitatively determined. As a result, 35.6 mg/ml of sorbitol remained and 13.2 mg/ml of 2KLGA and 6.2 mg/ml of idonic acid were generated. Sorbose was not detected.

25

Free text of Sequence Listing

- SEQ ID NO:3: Oligonucleotide designed to act as primer for sequencing insert DNA of pUCP19-HC.
- SEQ ID NO:4: Oligonucleotide designed to act as primer for sequencing insert DNA of pUCP19-HC.
- 5 SEQ ID NO:5: Oligonucleotide designed to act as primer for sequencing insert DNA of pUCP19-HC.
- SEQ ID NO:6: Oligonucleotide designed to act as primer for sequencing insert DNA of pUCP19-HC.
- SEQ ID NO:7: Oligonucleotide designed to act as primer for
10 sequencing insert DNA of pUCP19-HC.
- SEQ ID NO:8: Oligonucleotide designed to act as primer for sequencing insert DNA of pUCP19-HC.
- SEQ ID NO:9: Oligonucleotide designed to act as primer for sequencing insert DNA of pUCP19-HC.
- 15 SEQ ID NO:10: Oligonucleotide designed to act as primer for sequencing insert DNA of pUCP19-HC.
- SEQ ID NO:11: Oligonucleotide designed to act as primer for sequencing insert DNA of pUCP19-HC.
- SEQ ID NO:12: Oligonucleotide designed to act as primer for
20 sequencing insert DNA of pUCP19-HC.
- SEQ ID NO:13: Oligonucleotide designed to act as primer for sequencing insert DNA of pUCP19-HC.
- SEQ ID NO:14: Oligonucleotide designed to act as sense primer for amplifying DNA sequence encoding His-tagged SLDH and
25 promoter.
- SEQ ID NO:15: Oligonucleotide designed to act as antisense

11. A promoter gene comprising the DNA of the following (a)
or (b):

(a) a DNA having a base sequence of base numbers 1 - 536 of
the base sequence depicted in Sequence Listing SEQ ID NO:2

5 (b) a DNA having a base sequence of the above-mentioned (a)
wherein one to several bases is(are) deleted, substituted,
inserted, added or modified, which DNA shows a promoter
activity at least in one kind of microorganism.

10 12. A recombinant vector comprising a DNA of any of claims 6
to 9.

13. An expression vector comprising a DNA of any of claims 6
to 9.

15

14. The expression vector of claim 13 further comprising a
DNA encoding sorbose dehydrogenase and/or a DNA encoding
sorbosone dehydrogenase.

20 15. A transformant obtained by transforming a host cell with
an expression vector of claim 13 or 14.

16. The transformant of claim 15, which belongs to a genus
selected from the group consisting of *Escherichia coli*, the
25 genus *Pseudomonas*, the genus *Gluconobacter*, the genus
Acetobacter and the genus *Pseudogluconobacter*.

FIG. 1

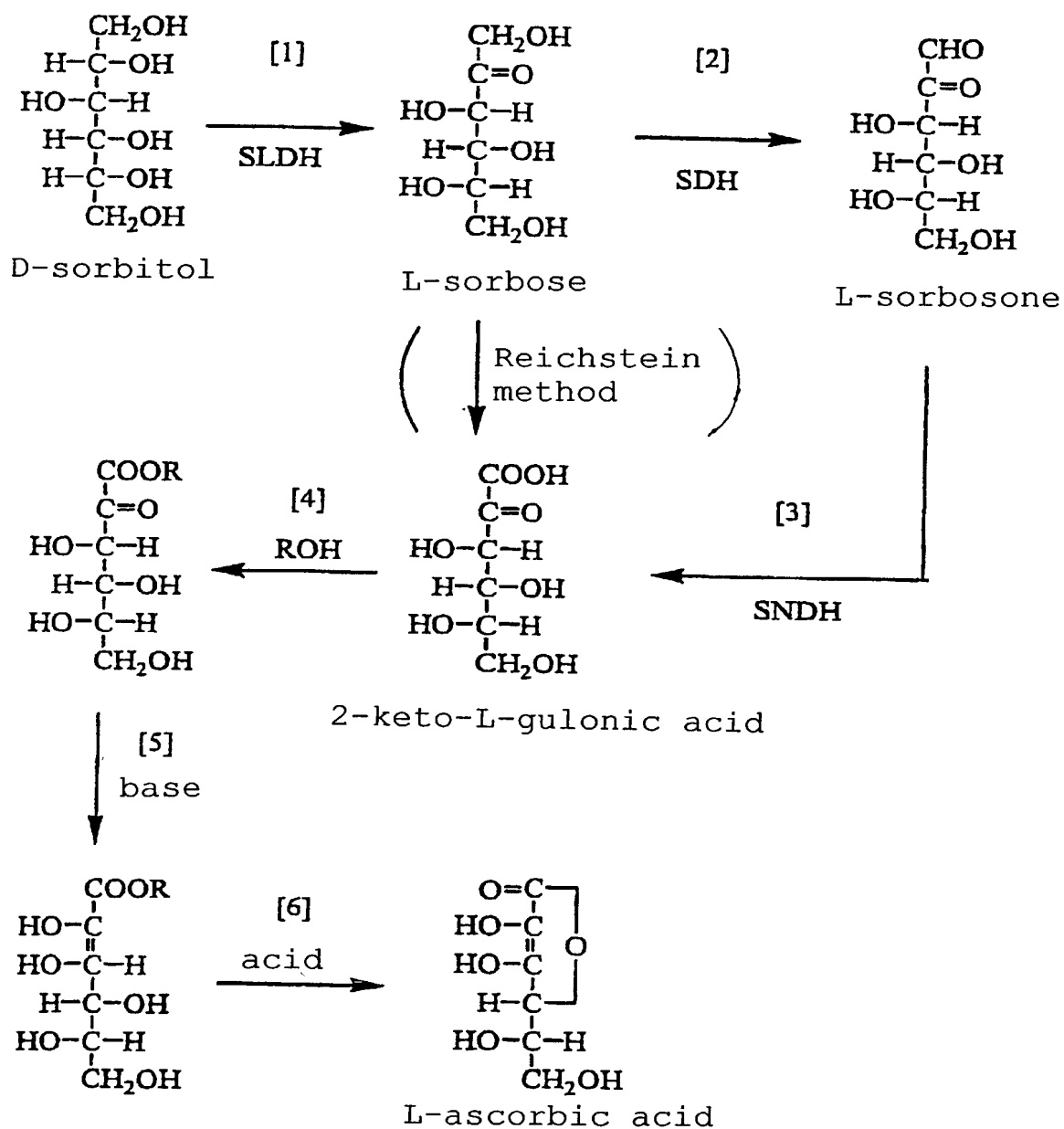


FIG. 2

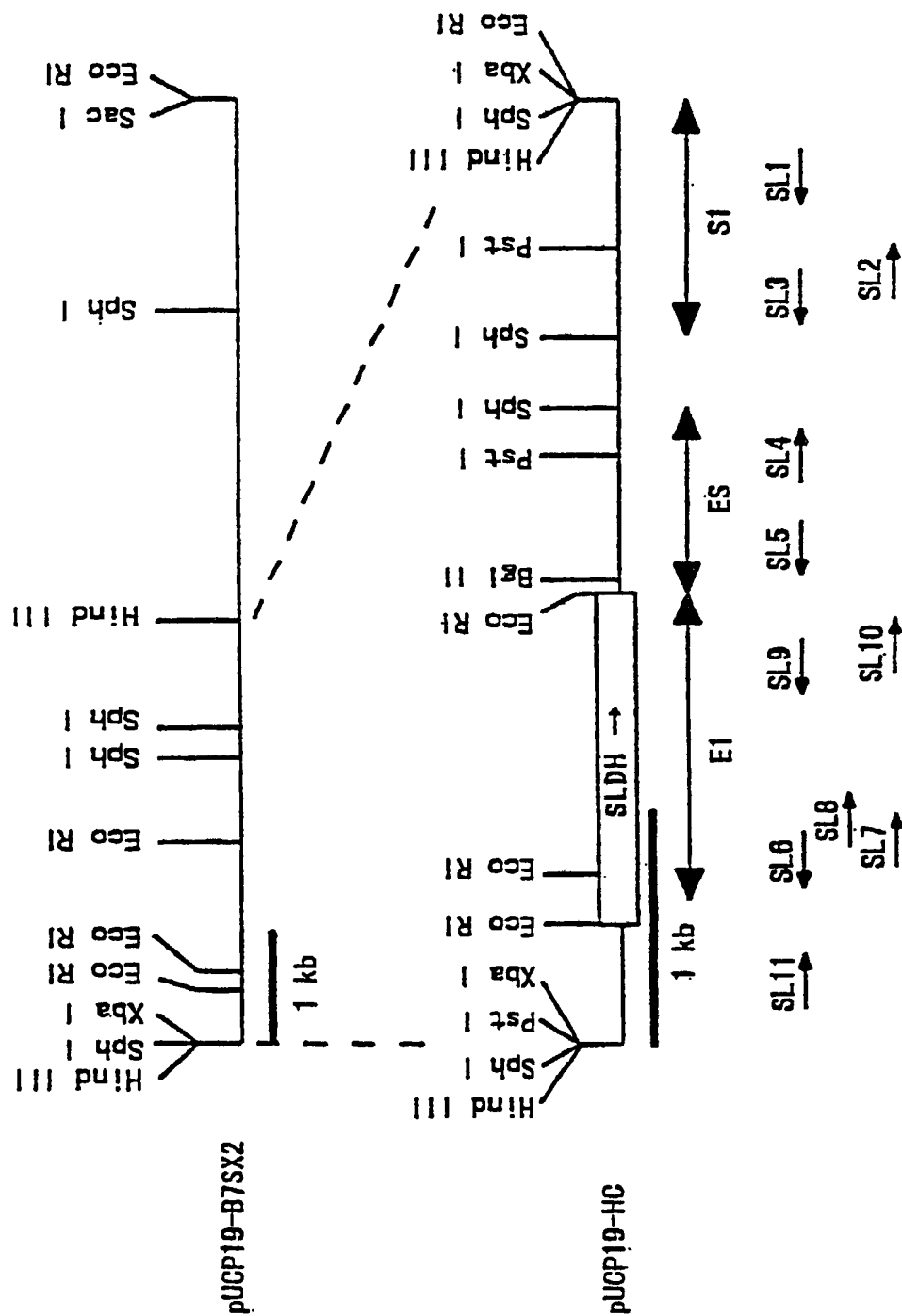


FIG. 3

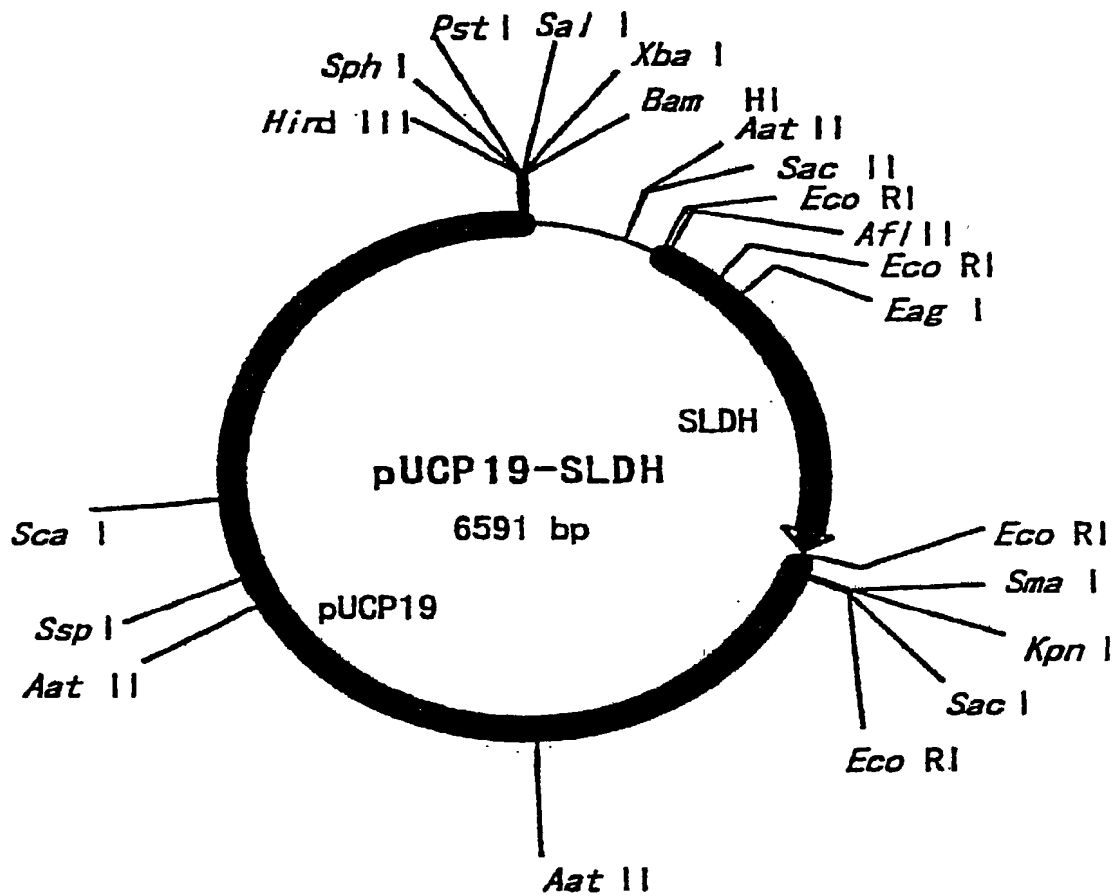


FIG. 4

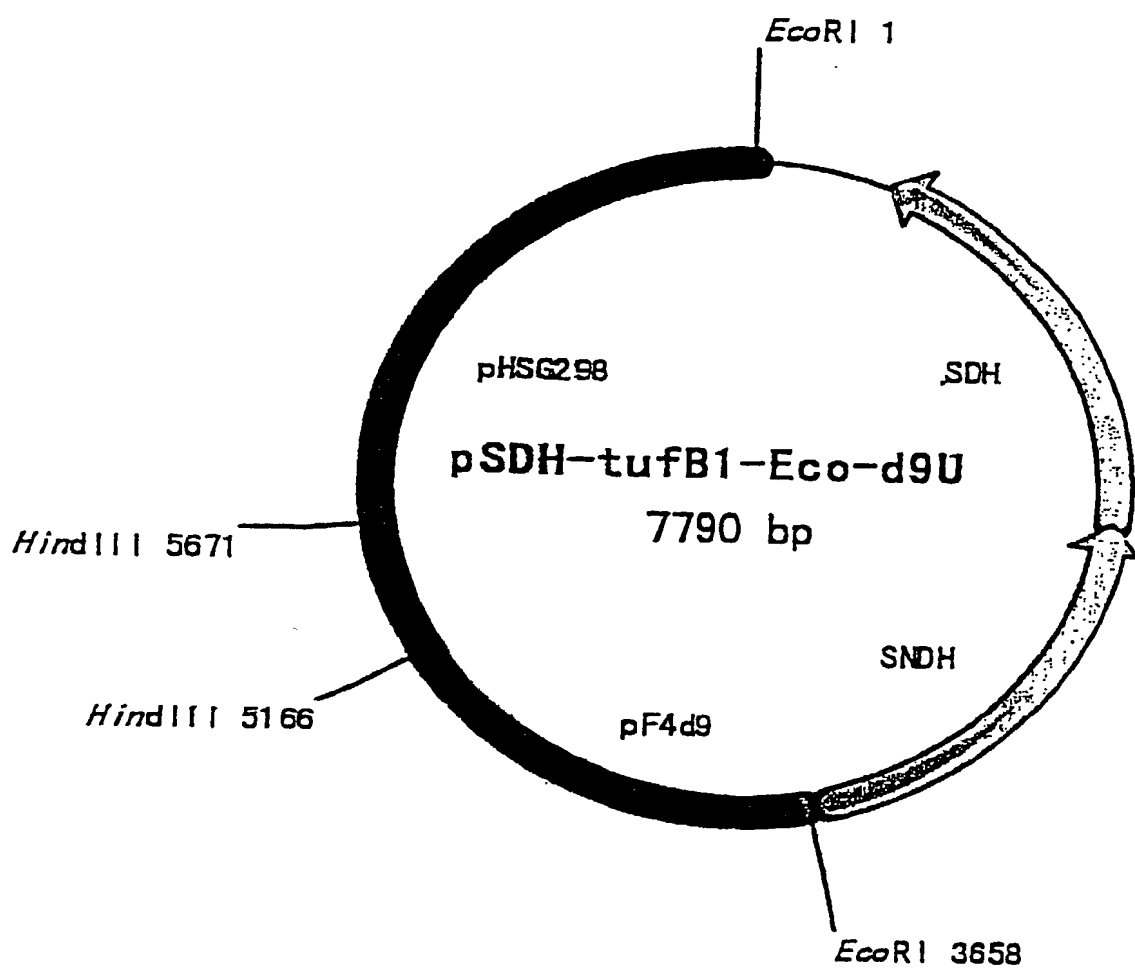


FIG. 5

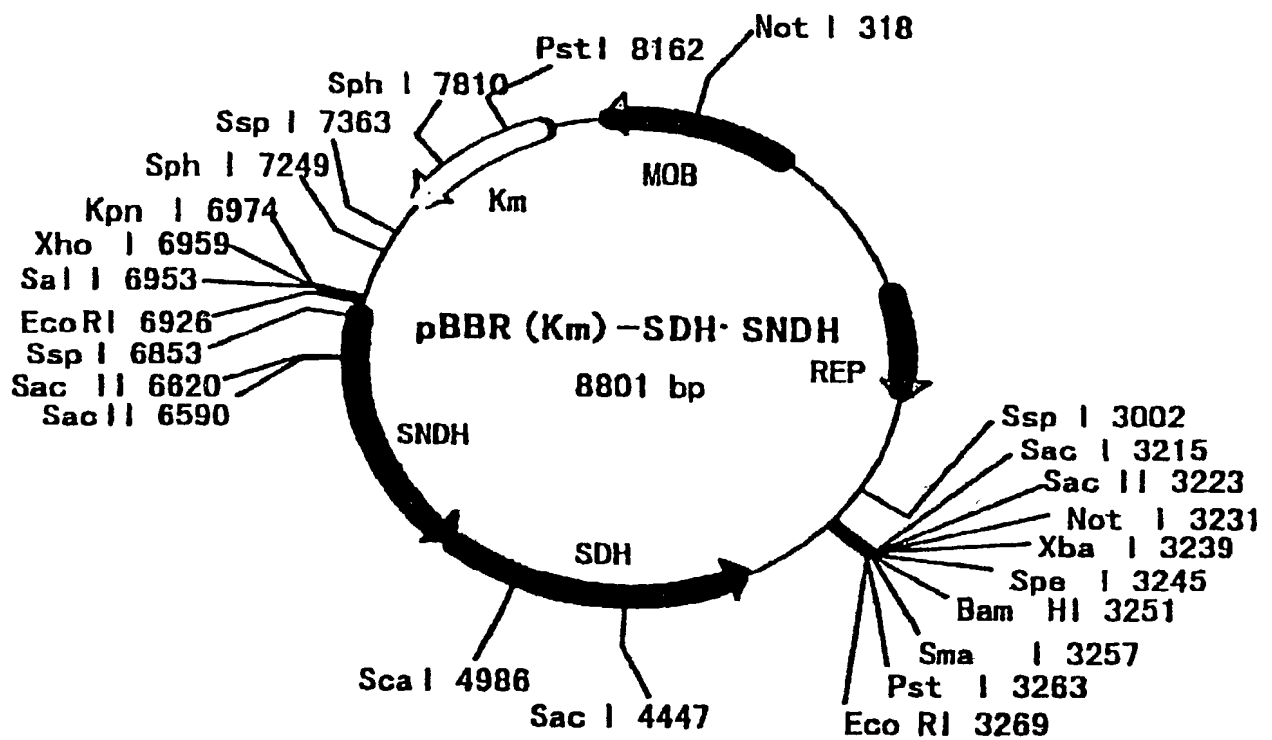


FIG. 6

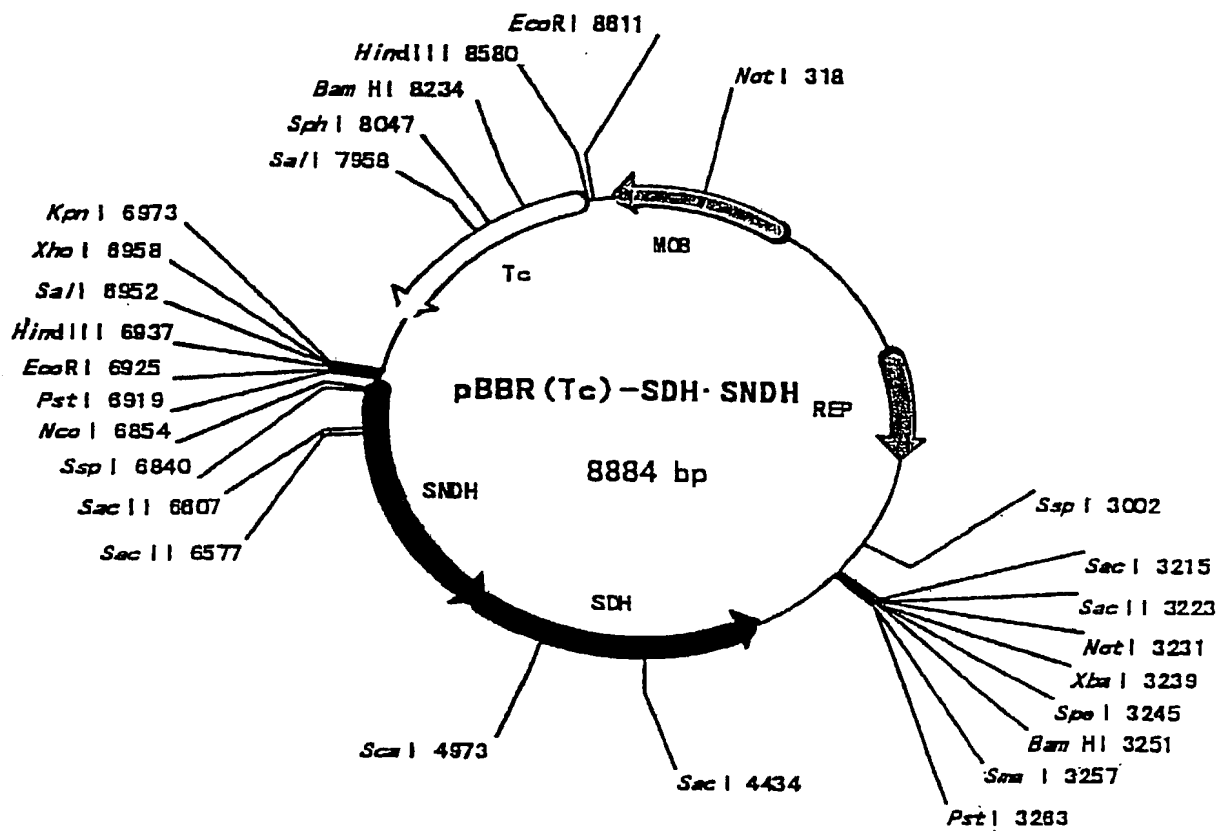
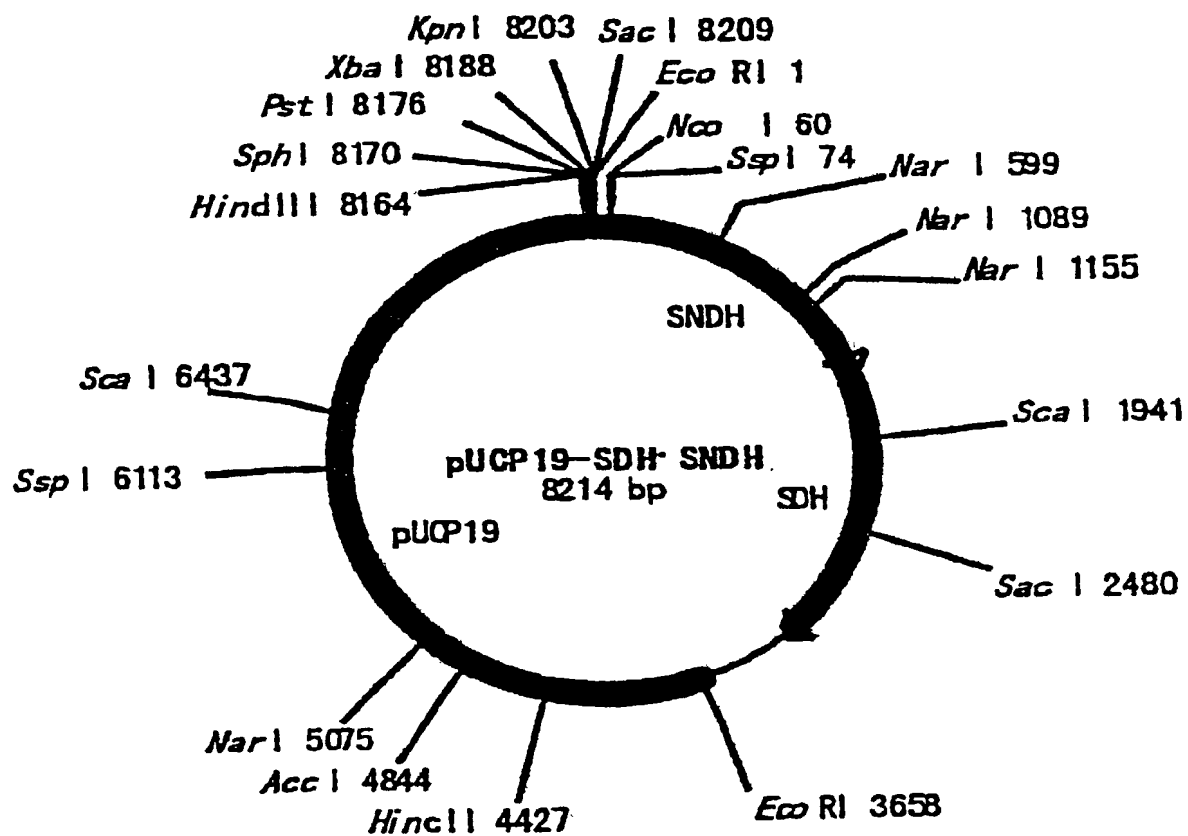


FIG. 7



Declaration, Power of Attorney and Petition

Page 1 of 4

WE (I) the undersigned inventor(s), hereby declare(s) that:

My residence, post office address and citizenship are as stated below next to my name,

We (I) believe that we are (I am) the original, first, and joint (sole) inventor(s) of the subject matter which is claimed and for which a patent is sought on the invention entitled

SORBITOL DEHYDROGENASE, GENE ENCODING THE SAME AND USE THEREOF

the specification of which

☐ is attached hereto.

☒ was filed on September 17, 2001 as
Application Serial No. 09/926,163
and amended on _____.

☒ was filed as PCT international application

Number PCT/JP00/01608

on March 16, 2000,

and was amended under PCT Article 19

on _____ (if applicable).

We (I) hereby state that we (I) have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

We (I) acknowledge the duty to disclose information known to be material to the patentability of this application as defined in Section 1.56 of Title 37 Code of Federal Regulations.

We (I) hereby claim foreign priority benefits under 35 U.S.C. § 119(a)-(d) or § 365(b,) of any foreign application(s) for patent or inventor's certificate, or § 365(a) of any PCT International application which designated at least one country other than the United States, listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate, or PCT International application having a filing date before that of the application on which priority is claimed. Prior Foreign Application(s)

Application No.	Country	Day/Month/Year	Priority Claimed
<u>72810/1999</u>	<u>Japan</u>	<u>17/03/1999</u>	<input checked="" type="checkbox"/> Yes <input type="checkbox"/> No
<u>224679/1999</u>	<u>Japan</u>	<u>06/08/1999</u>	<input checked="" type="checkbox"/> Yes <input type="checkbox"/> No
_____	_____	_____	<input type="checkbox"/> Yes <input type="checkbox"/> No
_____	_____	_____	<input type="checkbox"/> Yes <input type="checkbox"/> No

We (I) hereby claim the benefit under Title 35, United States Code, § 119(e) of any United States provisional application(s) listed below.

(Application Number)	(Filing Date)
(Application Number)	(Filing Date)

We (I) hereby claim the benefit under 35 U.S.C. § 120 of any United States application(s), or § 365(c) of any PCT International application designating the United States, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT International application in the manner provided by the first paragraph of 35 U.S.C. § 112, I acknowledge the duty to disclose information which is material to patentability as defined in 37 CFR § 1.56 which became available between the filing date of the prior application and the national or PCT International filing date of this application.

Application Serial No.	Filing Date	Status (pending, patented, abandoned)
PCT/JP00/01608	March 16, 2000	pending

And we (I) hereby appoint: Norman F. Oblon, Registration Number 24,618; Marvin J. Spivak, Registration Number 24,913; C. Irvin McClelland, Registration Number 21,124; Gregory J. Maier, Registration Number 25,599; Arthur I. Neustadt, Registration Number 24,854; Richard D. Kelly, Registration Number 27,757; James D. Hamilton, Registration Number 28,421; Eckhard H. Kuesters, Registration Number 28,870; Robert T. Pous, Registration Number 29,099; Charles L. Gholz, Registration Number 26,395; Vincent J. Sunderdick, Registration Number 29,004; William E. Beaumont, Registration Number 30,996; Steven B. Kelber, Registration Number 30,073; Robert F. Gnuse, Registration Number 27,295; Jean-Paul Lavalleye, Registration Number 31,451; Timothy R. Schwart, Registration Number 32,171; Stephen G. Baxter, Registration Number 32,884; Martin M. Zoltick, Registration Number 35,745; Robert W. Hahl, Registration Number 33,893; Richard L. Treanor, Registration Number 36,379; Steven P. Weihrouch, Registration Number 32,829; John T. Goolkasian, Registration Number 26,142; Marc R. Labgold, Registration Number 34,651; William J. Healey, Registration Number 36,160; Richard L. Chinn, Registration Number 34,305; Steven E. Lipman, Registration Number 30,011; Carl E. Schlier, Registration Number 34,426; James J. Kulbaski, Registration Number 34,648; Catherine B. Richardson, Registration Number 39,007; Richard A. Neifeld, Registration Number 35,299; J. Derek Mason, Registration Number 35,270; and Jacques M. Dulin, Registration Number 24,067; our (my) attorneys, with full powers of substitution and revocation, to prosecute this application and to transact all business in the Patent Office connected therewith; and we (I) hereby request that all correspondence regarding this application be sent to the firm of OBLON, SPIVAK, MCCLELLAND, MAIER & NEUSTADT, P.C., whose Post Office Address is: Fourth Floor, 1755 Jefferson-Davis-Highway, Arlington, Virginia 22202.

We (I) declare that all statements made herein of our (my) own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Takashi SHIBATA
NAME OF FIRST JOINT INVENTOR

Residence: Tsukuba-shi, Ibaraki, Japan

Takashi Shibata
Signature of Inventor

Citizen of: Japan

Post Office Address: 308, Anthimu-Tsukuba, 1-20-11,
Ninomiya, Tsukuba-shi, Ibaraki 305-0051 Japan

October 16, 2001

Date

5-00
Yoshimasa SAITO
NAME OF FIFTH JOINT INVENTOR

Yoshimasa Saito
Signature of Inventor

10/18/01
Date

Residence: Mitaka-shi, Tokyo, Japan

Citizen of: Japan

Post Office Address: 5-9-33, Mure, Mitaka-shi, Tokyo
181-0002 Japan JPX

6-00
Michio YAMASHITA
NAME OF SIXTH JOINT INVENTOR

Michio Yamashita
Signature of Inventor

Oct. 23, 2001
Date

Residence: Tsukuba-shi, Ibaraki, Japan

Citizen of: Japan

Post Office Address: 3-11-11, Namiki, Tsukuba-shi, Ibaraki
305-0044 Japan JPX

7-00
Yoko TAKATA
NAME OF SEVENTH JOINT INVENTOR

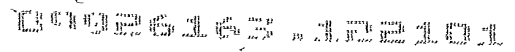
Yoko Takata
Signature of Inventor

Oct. 25, 2001
Date

Residence: Osaka-shi, Osaka, Japan

Citizen of: Japan

Post Office Address: 2-14-10-405, Nishinakajima,
Yodogawa-ku, Osaka-shi, Osaka 532-0011 Japan JPX



<110> SHIBATA, Takashi
ICHIKAWA, Chiyo
MATSUURA, Mitsutaka
NOGUCHI, Yuji
SAITO, Yoshimasa
YAMASHITA, Michio
TAKATA, Yoko

1

catgcccggc cttgtcggtc cccgtcagcg accggcccga aaccacggag aattcc atg															539	
															Met	1
att acg cgc gaa acc ctt aag tct ctt cct gcc aat gtc cag gct ccc															587	
Ile	Thr	Arg	Glu	Thr	Leu	Lys	Ser	Leu	Pro	Ala	Asn	Val	Gln	Ala	Pro	
			5					10					15			
ccc tat gac atc gac ggg atc aag cct ggg atc gtg cat ttc ggt gta															635	
Pro	Tyr	Asp	Ile	Asp	Gly	Ile	Lys	Pro	Gly	Ile	Val	His	Phe	Gly	Val	
		20					25					30				
ggt aac ttt ttt cga gcc cat gag gcg ttc tac gtc gag cag att ctt															683	
Gly	Asn	Phe	Phe	Arg	Ala	His	Glu	Ala	Phe	Tyr	Val	Glu	Gln	Ile	Leu	
	35					40					45					
gaa cac gct ccg gac tgg gcg att gtt ggt gtt ggc ctg acg ggc agt															731	
Glu	His	Ala	Pro	Asp	Trp	Ala	Ile	Val	Gly	Val	Gly	Leu	Thr	Gly	Ser	
50					55					60					65	
gac cgt tca aag aaa aaa gcc gag gaa ttc aag gcc cag gac tgc ctg															779	
Asp	Arg	Ser	Lys	Lys	Lys	Ala	Glu	Glu	Phe	Lys	Ala	Gln	Asp	Cys	Leu	
				70					75					80		
tat tcc ctg acc gag acg gct ccg tcc ggc aag agc acg gtg cgc gtc															827	
Tyr	Ser	Leu	Thr	Glu	Thr	Ala	Pro	Ser	Gly	Lys	Ser	Thr	Val	Arg	Val	
			85					90					95			
atg ggc gcg ctg cgt gac tat ctg ctt gcc ccg gcc gat ccg gaa gcc															875	
Met	Gly	Ala	Leu	Arg	Asp	Tyr	Leu	Leu	Ala	Pro	Ala	Asp	Pro	Glu	Ala	
		100					105					110				
gtg ctg aag cat ctt gtt gat ccg gcc atc cgc atc gtt tcc atg acg															923	
Val	Leu	Lys	His	Leu	Val	Asp	Pro	Ala	Ile	Arg	Ile	Val	Ser	Met	Thr	
	115					120					125					
atc acg gaa ggc ggc tac aac atc aac gag acg acc ggt gcg ttc gat															971	
Ile	Thr	Glu	Gly	Gly	Tyr	Asn	Ile	Asn	Glu	Thr	Thr	Gly	Ala	Phe	Asp	
130					135				140						145	
ctg gag aat gcg gca gta aag gcc gac ctc aag aac ccg gaa aag ccg															1019	
Leu	Glu	Asn	Ala	Ala	Val	Lys	Ala	Asp	Leu	Lys	Asn	Pro	Glu	Lys	Pro	
			150						155					160		
tct acc gtt ttc ggt tac gtg gtc gag gcc ctg cgt cgt cgt tgg gat															1067	
Ser	Thr	Val	Phe	Gly	Tyr	Val	Val	Glu	Ala	Leu	Arg	Arg	Arg	Trp	Asp	
			165					170					175			
gcc ggt ggt aag gca ttt acg gtc atg tcc tgt gat aac ctg cgt cat															1115	
Ala	Gly	Gly	Lys	Ala	Phe	Thr	Val	Met	Ser	Cys	Asp	Asn	Leu	Arg	His	
		180					185					190				
aac ggc aat gtc gcc cgc aag gcc ttc ctc ggc tat gcg aag gcg cgc															1163	
Asn	Gly	Asn	Val	Ala	Arg	Lys	Ala	Phe	Leu	Gly	Tyr	Gly	Ala	Lys	Ala	Arg
	195				200					205						

gat ccg gag ttg gcg aag tgg att gag gaa aac gcg acc ttc ccg aac Asp Pro Glu Leu Ala Lys Trp Ile Glu Glu Asn Ala Thr Phe Pro Asn 210 215 220 225	1211
gga atg gtt gat cgc atc acc ccg acc gtt tcg gcg gaa atc gcc aag Gly Met Val Asp Arg Ile Thr Pro Thr Val Ser Ala Glu Ile Ala Lys 230 235 240	1259
aag ctc aac gcg gcc agt ggg ctg gat gac gac ctg ccg ctg gtg gcc Lys Leu Asn Ala Ala Ser Gly Leu Asp Asp Asp Leu Pro Leu Val Ala 245 250 255	1307
gag gat ttc cat cag tgg gtg ctg gaa gac cag ttt gcg gat ggc cgt Glu Asp Phe His Gln Trp Val Leu Glu Asp Gln Phe Ala Asp Gly Arg 260 265 270	1355
ccg ccg ctt gaa aaa gcc ggc gtg cag atg gtc ggg gac gtg acg gac Pro Pro Leu Glu Lys Ala Gly Val Gln Met Val Gly Asp Val Thr Asp 275 280 285	1403
tgg gag tac gtc aag atc cga atg ctc aat gca ggg cat gtc atg ctc Trp Glu Tyr Val Lys Ile Arg Met Leu Asn Ala Gly His Val Met Leu 290 295 300 305	1451
tgc ttc cca ggc att ctg gtc ggc tat gag aat gtg gat gac gcc att Cys Phe Pro Gly Ile Leu Val Gly Tyr Glu Asn Val Asp Asp Ala Ile 310 315 320	1499
gaa gac agc gaa ctc ctt ggc aat ctg aag aac tat ctc aac aag gat Glu Asp Ser Glu Leu Leu Gly Asn Leu Lys Asn Tyr Leu Asn Lys Asp 325 330 335	1547
gtc atc ccg acc ctg aag gcg cct tca ggc atg acg ctc gaa ggc tat Val Ile Pro Thr Leu Lys Ala Pro Ser Gly Met Thr Leu Glu Gly Tyr 340 345 350	1595
cgg gac agc gtc atc agc cgt ttc tcc aac aag gcg atg tcg gac cag Arg Asp Ser Val Ile Ser Arg Phe Ser Asn Lys Ala Met Ser Asp Gln 355 360 365	1643
acg ctc cgg att gct agc gat ggc tgt tcc aag gtt cag gtg ttc tgg Thr Leu Arg Ile Ala Ser Asp Gly Cys Ser Lys Val Gln Val Phe Trp 370 375 380 385	1691
acg gaa acc gtg cgt cgg gcg atc gaa gac aag cgg gac ctg tca cgt Thr Glu Thr Val Arg Arg Ala Ile Glu Asp Lys Arg Asp Leu Ser Arg 390 395 400	1739
ata gcg ttc gga att gca tcc tat ctc gaa atg ctg cgt ggt cgc gac Ile Ala Phe Gly Ile Ala Ser Tyr Leu Glu Met Leu Arg Gly Arg Asp 405 410 415	1787
gag aag ggc ggg acg tat gaa tcg tcc gag ccg act tat ggc gac gcc Glu Lys Gly Gly Thr Tyr Glu Ser Ser Glu Pro Thr Tyr Gly Asp Ala 420 425 430	1835
gaa tgg aag ttg gcc aag gcg gac gac ttc gaa agc tct ctg aag ctc	1883

Glu Trp Lys Leu Ala Lys Ala Asp Asp Phe Glu Ser Ser Leu Lys Leu	435	440	445	
ccg gcg ttc gat ggg tgg cgc gat ctg gat acg tcc gaa ctg gat caa				1931
Pro Ala Phe Asp Gly Trp Arg Asp Leu Asp Thr Ser Glu Leu Asp Gln	450	455	460	465
aag gtc atc gtg ctg cgg aag atc atc cgc gaa aag ggc gta aaa gcc				1979
Lys Val Ile Val Leu Arg Lys Ile Ile Arg Glu Lys Gly Val Lys Ala	470	475	480	
gcc atc ccg gcc tga attcggcttt tagggtagcg actgaaacag aaaaccgcgc				2034
Ala Ile Pro Ala	485			
tctggaagga gcgcggtttt ttttatgctc agatctgtcc catcaggaca aggatcacga				2094
cgaccacgat caggacaagt ccgctggagg gggagcccca ttctgaactg tacggccatg				2154
acggcagcgc accgagatca ggattacaag aaggatcagt cccatggcac atctctcttg				2214
ccggttgaga ctggtctgtg ttccgggtgt ctaaaaagtt tccgtagggg cgcgaaagat				2274
caaagctgtc ggtcgcgctt aatccggtcc caagccgcat tgatgcgggc cacccggtcc				2334
tgtgcgcgctt tgcgctctgt ctctgacata ggtttctggg ccagcacgtc cggatgatgt				2394
tcgcggatca ggggtgcgcca gcgcacgcgg atttctgtgt cagttgcgct gcgggtgatg				2454
ccgagaatac gataggcatc cggtctggtt ccgctggcgg cgcgattggt gccgctttcg				2514
gcccgggtccc atgctcctgg cggcaggcca aatgccccgt gaacgcgctg cagaaaatcg				2574
atttccttcg ggtgaagctc gcggctgggg ccggcatcgg cacgggcgat acggaacagt				2634
gccgtcatga ggttctcaag cggcgccgta ttatcggcat aggccttgcc catttcgcgg				2694
gcatacatct cgaaatcgtc cgtccggtcg cgggcgcgat cgaacagcat gccgacttcc				2754
ttggtgttat cgggggggaa ctggaagcag gtcttgaaag cgttgatttc gtgtcggttc				2814
accggcccgt cgatcttcgc cagcttcgcg cacagggcaa caaggccgat ggcgtaaagc				2874
tgatctcggt tgcccagggc cgcagcaatc ttggcagcgc cgaaaaaggc cgcgctgttg				2934
ggatcgggac ggccattcgc gggaaagcgc tcactccagc cgcccgttga gggcttgagt				2994
agcgaaccgt tatcggcggc atgccccagc gctgcgccca tcagtgtctc gaaaggacca				3054
ccaaccgcga agcccgcgac accaccgaac atcttgcccc agatagccat gtcataacc				3114
tagcacgccc gctcacagcg gcaaatgaca gatcgcaggc taggtgtagg tgctgatgcg				3174
ccaaccgccc gggcttgcgg tgtggtagaa gctaggagtt acgaacttat cgctgtctca				3234
tgcttttgag gcgcaggttc ttctgttcgt ttcatgacgg atatttttat gcccaccttg				3294

atccagactg ctacttcgat ccctttccgc tctgatgacg aactgatgga tcttttgatc 3354
aagcgtctgc caatgtggct gcagaaagtg ctgaactggc tgcgggaagc ggatcataaa 3414
tgggttcgga ttccggcggg cgtgctgttc atgctgggag gcgttctgtc catcctgcct 3474
gttctgggtc tgtggatgct gccggtcggc gtgatgttgc ttgctcagga tattccgttc 3534
ttccgtcgcc ttccaggccg cctcttgcgc tggatcgaac gtcaacatcc ggattggctg 3594
ggccttccgg cgaaaagcgg cagaagctaa ccgttcgtct ggacgtgttt ctgaagatgt 3654
gtcagtgtgc caaccgcag ggctgaagcc agtgggagct ctggtggtgc cgcggcatgc 3714
agagaagcca ccagagacgc aaagctctgc tggcggactg cggccatgc gtccagtata 3774
gcccagaact cgggttcag tgccacggac gtccggtgtc ctgacagaga caggctgcgt 3834
ttgacgagat cactcattcc ggttgtttct caaggcgctt caaagccat tgtgcggttt 3894
cggaacatc agggtcgga tcaactcagc gctccgcgc agaagatata agcgacggat 3954
cggccgagtt gccgatcgc atcaggacag ttacgtacga accggttgcg tccaatccgt 4014
ttgaccggag agccagaaaa aaacgtccgg aatgtcgcat tatccagccg caccagttcg 4074
tcgagttttg gtgcaatcag ctccgggagg gcctgaagct t 4115

<210> 2
<211> 485
<212> PRT
<213> Gluconobacter oxydans

<400> 2

Met Ile Thr Arg Glu Thr Leu Lys Ser Leu Pro Ala Asn Val Gln Ala
1 5 10 15

Pro Pro Tyr Asp Ile Asp Gly Ile Lys Pro Gly Ile Val His Phe Gly
20 25 30

Val Gly Asn Phe Phe Arg Ala His Glu Ala Phe Tyr Val Glu Gln Ile
35 40 45

Leu Glu His Ala Pro Asp Trp Ala Ile Val Gly Val Gly Leu Thr Gly
50 55 60

Ser Asp Arg Ser Lys Lys Lys Ala Glu Glu Phe Lys Ala Gln Asp Cys
65 70 75 80

Leu Tyr Ser Leu Thr Glu Thr Ala Pro Ser Gly Lys Ser Thr Val Arg

85	90	95
Val Met Gly Ala Leu Arg Asp Tyr	Leu Leu Ala Pro Ala Asp Pro Glu	
100	105	110
Ala Val Leu Lys His Leu Val Asp Pro Ala Ile Arg Ile Val Ser Met		
115	120	125
Thr Ile Thr Glu Gly Gly Tyr Asn Ile Asn Glu Thr Thr Gly Ala Phe		
130	135	140
Asp Leu Glu Asn Ala Ala Val Lys Ala Asp Leu Lys Asn Pro Glu Lys		
145	150	155
Pro Ser Thr Val Phe Gly Tyr Val Val Glu Ala Leu Arg Arg Arg Trp		
165	170	175
Asp Ala Gly Gly Lys Ala Phe Thr Val Met Ser Cys Asp Asn Leu Arg		
180	185	190
His Asn Gly Asn Val Ala Arg Lys Ala Phe Leu Gly Tyr Ala Lys Ala		
195	200	205
Arg Asp Pro Glu Leu Ala Lys Trp Ile Glu Glu Asn Ala Thr Phe Pro		
210	215	220
Asn Gly Met Val Asp Arg Ile Thr Pro Thr Val Ser Ala Glu Ile Ala		
225	230	235
Lys Lys Leu Asn Ala Ala Ser Gly Leu Asp Asp Asp Leu Pro Leu Val		
245	250	255
Ala Glu Asp Phe His Gln Trp Val Leu Glu Asp Gln Phe Ala Asp Gly		
260	265	270
Arg Pro Pro Leu Glu Lys Ala Gly Val Gln Met Val Gly Asp Val Thr		
275	280	285
Asp Trp Glu Tyr Val Lys Ile Arg Met Leu Asn Ala Gly His Val Met		
290	295	300
Leu Cys Phe Pro Gly Ile Leu Val Gly Tyr Glu Asn Val Asp Asp Ala		
305	310	315
		320

Asp Val Ile Pro Thr Leu Lys Ala Pro Ser Gly Met Thr Leu Glu Gly
340 345 350

Tyr Arg Asp Ser Val Ile Ser Arg Phe Ser Asn Lys Ala Met Ser Asp
355 360 365

Gln Thr Leu Arg Ile Ala Ser Asp Gly Cys Ser Lys Val Gln Val Phe
370 375 380

Trp Thr Glu Thr Val Arg Arg Ala Ile Glu Asp Lys Arg Asp Leu Ser
385 390 395 400

Arg Ile Ala Phe Gly Ile Ala Ser Tyr Leu Glu Met Leu Arg Gly Arg
405 410 415

Asp Glu Lys Gly Gly Thr Tyr Glu Ser Ser Glu Pro Thr Tyr Gly Asp
420 425 430

Ala Glu Trp Lys Leu Ala Lys Ala Asp Asp Phe Glu Ser Ser Leu Lys
435 440 445

Leu Pro Ala Phe Asp Gly Trp Arg Asp Leu Asp Thr Ser Glu Leu Asp
450 455 460

Gln Lys Val Ile Val Leu Arg Lys Ile Ile Arg Glu Lys Gly Val Lys
465 470 475 480

Ala Ala Ile Pro Ala
485

```
<210> 3
<211> 16
<212> DNA
<213> Artificial Sequence
```

<220>
<223> synthetic DNA

<400> 3
gctgctgagt gatccg

[illegible]

<400> 8
gatgcttcag cacggc

16

```
<210> 9
<211> 16
<212> DNA
<213> Artificial Sequence
```

<220>
<223> synthetic DNA

```
<400> 9
gacgatcacg gaaggc
```

16

```
<210> 10
<211> 16
<212> DNA
<213> Artificial Sequence
```

<220>
<223> synthetic DNA

```
<400> 10
ggttacgtgg tcgacg
```

16

```
<210> 11
<211> 17
<212> DNA
<213> Artificial Sequence
```

<220>
<223> synthetic DNA

```
<400> 11
ctatacctga caggtcc
```

17

```
<210> 12
<211> 16
<212> DNA
<213> Artificial Sequence
```

<220>
<223> synthetic DNA

```
<400> 12
gcgcgatctg gatacg
```

16

<210>	13
<211>	16
<212>	DNA
<213>	Artificial Sequence

<220>
 <223> synthetic DNA
 <400> 13
 cgaggatctc gaacgg 16

<210> 14
 <211> 18
 <212> DNA
 <213> Artificial Sequence

<220>
 <223> synthetic DNA
 <400> 14
 cggattgcta gcgatggc 18

<210> 15
 <211> 47
 <212> DNA
 <213> Artificial Sequence

<220>
 <223> synthetic DNA
 <400> 15
 atcgaggatc ctcaatgatg atgatgatga tgggccggga tggcggc 47

<210> 16
 <211> 30
 <212> DNA
 <213> Artificial Sequence

<220>
 <223> synthetic DNA
 <400> 16
 atcgaggatc cattcggctt ttagggtagc 30

<210> 17
 <211> 28
 <212> DNA
 <213> Artificial Sequence

<220>
 <223> synthetic DNA
 <400> 17
 tagctgagct catgggacag atctgagc 28

<210> 18
 <211> 10

[illegible]

<400> 18

Met Ile Thr Arg Glu Thr Leu Lys Ser Leu
1 5 10

```
<210> 19
<211> 33
<212> DNA
<213> Artificial Sequence
```

<220>
<223> synthetic DNA

```
<400> 19
taggaatatt tctcatgatt acgcgcgaaa ccc
```

```
<210> 20
<211> 16
<212> DNA
<213> Artificial Sequence
```

<220>
<223> synthetic DNA

```
<400> 20
gatgcttcag cacggc
```